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INFLUENCE OF BIOGENIC AMINES ON BOVINE LUTEAL STEROIDOGENESIS

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INFLUENCE OF BIOGENIC AMINES ON BOVINE LUTEAL STEROIDOGENESIS

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DISSERTATION

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DRUG DESCRIPTION

2-Amino-6,7-dihydroxy-1,2,3,4,-tetranapthalene hydrobromide	nonspecific dopamine receptor agonist
Butoxamine	β_2-adrenergic receptor antagonist
8-Hydroxydipropylamino	specific serotonin-1 receptor agonist
3-Isobutyl-methylxanthine (IBMX)	phosphodiesterase inhibitor
Isoproterenol	nonspecific β-adrenergic receptor agonist
Ketanserin	specific serotonin-2 receptor antagonist
Mianserin	nonspecific serotonin receptor antagonist
Phentolamine	nonspecific α-adrenergic receptor antagonist
Practolol	β_1-adrenergic receptor antagonist
Propranolol	nonspecific β-adrenergic receptor antagonist
Salbutamol	β_2-adrenergic receptor agonist
Terbutaline	β_2-adrenergic receptor agonist

ABSTRACT

Influence of Biogenic Amines on Bovine
Luteal Steroidogenesis

by

Paul Jeffrey Battista
University of New Hampshire, May 1987

To investigate the role of catecholamines and biogenic amines in the control of bovine luteal steroidogenesis, studies were conducted utilizing collagenase-dispersed luteal cells in a short-term incubation system. Studies designed to assess the role of catecholamines showed that the stimulatory response to the primary catecholamines norepinephrine and epinephrine was mediated by both β_1- and β_2-adrenergic receptors. The alternative pathway catecholamines octopamine, synephrine and deoxyepinephrine enhanced basal progesterone production, and this response was mediated by β-adrenergic receptors. Analysis of luteal tissue using high performance liquid chromatography showed nondetectable levels of norepinephrine and epinephrine. The *in vivo* administration of norepinephrine and epinephrine significantly stimulated luteal steroidogenesis without altering circulating levels of luteinizing hormone.

The addition of serotonin or 5-methoxytryptamine to luteal cell incubations increased the production of progesterone in a dose-dependent manner. The response to serotonin was mediated by a serotonin-1 receptor, which may not be associated with adenylate cyclase. Analysis of bovine luteal tissue using high performance liquid chromatography demonstrated the presence of serotonin, and showed that the concentration of serotonin varied with the stage of luteal development. Administration of serotonin *in vivo* resulted in significantly elevated levels of plasma progesterone, which were independent of changes in luteinizing hormone.

These results support the concept that a variety of biogenic amines influence bovine luteal steroidogenesis.

REVIEW OF THE LITERATURE

Neural Control of Reproductive Function

Most, if not all, reproductive functions are controlled by hormonal and neural influences. While the hormonal control of reproductive function has received the most attention (reviewed by Hansel et al., 1973; Niswender et al., 1985) the nervous system has been implicated in controlling a variety of reproductive processes including: gonadotropin secretion (reviewed by Naumenko, 1985), cyclicity (Hill, 1949, 1962; Gelderd and Pepper, 1979), prepuberal ovarian development (Aguado et al., 1982; Aguado and Ojeda, 1984a; Ben-Johnathan et al., 1984), follicular development (Brink and Grob, 1972; Grob, 1974; Burden, 1978; Curry et al., 1983), ovulation (reviewed by Bahr et al., 1974), ovarian steroidogenesis (Condon and Black, 1976; Godkin et al., 1977; Kawakami et al., 1981; Weiss et al., 1982), compensatory ovarian hypertrophy (Gerendai et al., 1978; Moger and Anakwe, 1986), pregnancy (reviewed by Burden, 1978), parturition (reviewed by Burden, 1978; Maitter, 1984; Legrand and Maitter, 1986), uterine and oviductal motility (reviewed by Black, 1979), uterine and ovarian blood flow (reviewed by Ford, 1985), puberty (reviewed by Foster et al., 1985), and seasonal reproduction (reviewed by Lincoln, 1984).

The following review of the literature is concerned with the relationship between the peripheral nervous system and the regulation of ovarian function. More specifically, the major focus of the following

literature review and the subsequent studies performed, is concerned with elucidating the role of biogenic amines in the control of steroidogenesis by the corpus luteum.

Neural Innervation of the Mammalian Reproductive Tract and Ovary

The mammalian reproductive tract is innervated by both sympathetic and parasympathetic components of the autonomic nervous system (reviewed by Bell, 1972; Black, 1974, 1979). Both noradrenergic and cholinergic nerve fibers innervate the vascular and nonvascular smooth muscle of the oviducts, uterus, cervix and vagina (reviewed by Papka et al., 1985). The sympathetic component of the autonomic nervous system innervating the reproductive tract arises from the hypogastric nerves (Brudin, 1965; Sjoberg, 1967; Marshall, 1970; Bell, 1972) while parasympathetic innervation arises from the pelvic nerve (Schofield, 1952; Carlson and DeFeo, 1965; Marshall, 1970). The mammalian oviduct also receives sympathetic innervation via the inferior, middle and superior ovarian nerves (Mitchell, 1938; Marshall, 1970; Bell, 1972). The reproductive system is more highly innervated by noradrenergic than cholinergic nerve fibers (Owman, 1967; Sjoberg, 1967; Black, 1974,1979). The distribution of noradrenergic and cholinergic nerve fibers supplying the reproductive tract follows a similar pattern across mammalian species, although the degree of innervation varies widely among species (reviewed by Black, 1974, 1979; Papka et al., 1985).

As in the reproductive tract, the ovary is innervated by both noradrenergic and cholinergic nerve fibers (reviewed by Bahr et al., 1974). The ovary receives sympathetic innervation from two main sources, the superior ovarian nerve, which travels along the suspensory ligament and the plexus nerve which accompanies the ovarian artery in

the ovarian plexus (Lawrence and Burden, 1980). Nerve fibers of the ovarian plexus terminate primarily on blood vessels while those of the superior ovarian nerve terminate on blood vessels and the steroid producing cells of the interstitial gland (Lawrence and Burden, 1980). Sympathetic nerve fibers arising from the superior ovarian nerve innervate the theca but not the granulosa cell layer of ovarian follicles (Burden, 1972; Unsicker, 1974; Walles, 1975, 1978; Stefenson et al., 1981). In addition, the corpus luteum is devoid of noradrenergic innervation (Burden, 1972; Unsicker, 1974; Black, 1979).

Cholinergic innervation of the mammalian ovary appears to arise from the vagus nerve (Hill, 1962; LePere et al., 1966; Burden and Lawrence, 1978). but parasympathetic innervation from the hypogastric (Stohr, 1957) and ovarian plexus (Burden et al., 1978) has also been demonstrated. Cholinergic innervation of the ovary is less developed but has the same distribution as noradrenergic fibers innervating blood vessels, ovarian stroma and the follicular wall, but not the corpus luteum (Jacoborwitz and Wallach. 1967; Owman et al., 1975; Walles et al., 1976; Burden and Lawrence, 1978; Black, 1979; Stefenson et al., 1981). As in the reproductive tract, the distribution of sympathetic and parasympathetic nerve fibers supplying the ovary tends to follow a similar pattern among species. although the degree of innervation does vary (Bahr et al., 1974; Burden, 1979; Stefenson et al., 1981).

Recently a variety of other biogenic amines and neuroactive peptides have been observed in mammalian reproductive organs. These compounds include dopamine (Farrar et al., 1980; Van Orden et al., 1980; Bahr and Ben-Johnathan 1981; Ben-Johnathan et al., 1984), epinephrine (Wurtman et al., 1963; Van Orden et al., 1980; Bahr and Ben-Johnathan 1985).

serotonin (Clausell and Soliman, 1978; Battista et al., 1987a), substance P (Makris et al., 1982; Dees et al., 1985; Ojeda et al., 1985; Papka et al., 1985), vasoactive intestinal peptide (Larsson et al., 1977; Alm et al., 1980a, b; Hakanson et al, 1982), gamma-aminobutyric acid (Del Rio and Caballero, 1980; Del Rio, 1981; Erdo et al., 1982; Schaffer and Hsueh, 1982), β-endorphin opiates (Tsong et al., 1982a,b; Alm et al., 1983; Lim et al., 1983; Lolait et al, 1985), neuropeptide tyrosine (Sternquist et al., 1983; Traurig et al., 1984; Papka et al., 1985), neuropeptide Y (Sternquist et al., 1983; Blank et al., 1986), oxytocin, oxytocin-associated neurophysin, vasopressin (Wathes and Swann, 1982; Wathes et al., 1983; Wathes, 1983), and relaxin (reviewed by Sherwood and Downing, 1983).

The role of these compounds, as well as norepinephrine and acetylcholine, in the control of ovarian steroidogenesis will be addressed in a subsequent portion of this literature review.

THE CORPUS LUTEUM

The mammalian corpus luteum (CL) is a transient endocrine gland which was first observed by Volcherus Coiter in 1573 and later named as such by Marcello Malpighi in 1697 due to its yellow appearance in the cow. The early studies of Fraenkel and Cohn (1901) established that the CL was essential for maintenance of pregnancy in rabbits and that it appeared to meet the criteria of being an endocrine gland. In 1917 Loeb reported that guinea pigs required a CL for ova to induce development of the placenta. Later, Corner and Allen (1929,1930) demonstrated that an active principle could be obtained from the CL and the term progestin

was used to designate this lipid-soluble substance. The structure of progesterone was later determined by Slotta et al. (1934). Subsequent research has sought to describe the hormonal control of CL function.

Formation of the CL is dependent on the morphological and biochemical transformation of follicular theca and granulosa cells into luteal cells (Gier and Marion, 1961; Donaldson and Hansel, 1965a). This transformation of follicular cells into luteal cells is dependent upon the preovulatory surge of the pituitary gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH) (reviewed by Channing et al., 1980). In the cow, the weight and progesterone (P_4) content of the CL increase rapidly between days 3 and 12 of the estrous cycle (estrus=day 0) and remain relatively constant until day 16 (Erb et al., 1971). At this time luteolysis, or regression of the CL, occurs followed by follicular recruitment, ovulation and subsequent CL development (reviewed by Hansel et al., 1973).

Two distinct populations of luteal cells have been identified in a variety of species such as the pig (Corner, 1919), cow (Foley and Greenstein, 1958), human (Gillim et al., 1969), sheep (Warbritton, 1934), rat (Wilkinson et al., 1976), white-tailed deer (Sinha et al., 1971), white whale (Mossman and Duke, 1973), and rhesus monkey (Gulyas et al., 1979). There is contradictory evidence on the follicular origin of these two cell types, designated small and large luteal cells. Several histological studies on the formation of the bovine CL suggest that luteal cells are dervived from both cell types of the follicle, the granulosa giving rise to large luteal cells and the theca cells to the small luteal cells (McNutt, 1924; Foley and Greenstein, 1958; Gier and Marion, 1961; Donaldson and Hansel, 1965b). However, in the ewe and cow

it has been suggested that some of these large cells arise from small cells (Warbritton, 1934; Donaldson and Hansel, 1965b, Fitz et al., 1982). Recently, a study utilizing monoclonal antibodies directed against antigenic determinants of small and large luteal cells was conducted to determine the contribution of follicular cells to bovine luteal composition (Alila and Hansel, 1984). These authors reported that large cells were derived from granulosa while most of the small cells were of thecal origin, and that some small cells developed into large cells as the age of the CL increased. Additionally, they observed that granulosa-derived cells disappeared during early pregnancy while cells of thecal origin persisted throughout pregnancy.

Morphologically, large luteal cells are generally spherical in shape and their diameter greater than 20 um, while small luteal cells have a stellate appearance and have a cell diameter of 10-20 um (Priedkalns and Weber, 1968; Koos and Hansel, 1981; Fitz et al., 1982). Ultrastructurally, both small and large luteal cells contain abundant endoplasmic reticulum and numerous mitochondria (Niswender et al., 1985); features which are characteristic of steroidogenically active cells (Christensen and Gillim, 1969; Savard, 1973). Large luteal cells also have features which are consistent with a protein secreting function, such as numerous Golgi complexes, rough endoplasmic reticulum and secretory granules (Niswender et al., 1985). These electron dense secretory granules are not characteristically found in small luteal cells although these cells do contain large numbers of lipid droplets (O'Shea et al., 1979; Koos and Hansel, 1981; Chengini et al., 1984; Rodgers et al., 1984; Niswender et al., 1985).

Although the follicular origin of small and large luteal cells is

controversial, the distinct steroidogenic capacities of these two cell types is more widely accepted. Differences in steroidogenic activity between small and large luteal cells was first reported by Leman and Loir (1977). They found that the basal rate of P_4 secretion by large cells was greater than that of small cells, and small cells were more responsive to LH. Similar results have now been reported utilizing luteal cell preparations from the cow (Ursely and Leymarie. 1979; Koos and Hansel, 1981), ewe (Fitz et al., 1982; Rodgers et al., 1983), and rabbit (Hoyer et al., 1986). Thus, both large and small cells secrete P_4, but the regulation of steroidogenesis in the two cell types appears to be quite different. In the unstimulated state, large luteal cells secrete approximately 20 times as much P_4 as do small cells (Fitz et al., 1982). That LH can stimulate P_4 production in small cells is consistent with the finding that small cells contain more receptors for LH than do large cells (Fitz et al., 1982). In contrast. receptors for prostaglandins (Fitz et al., 1982) and estradiol (Glass et al., 1984) are more abundant on large cells. In addition to LH. steroidogenesis in small cells can be stimulated by dibutyryl cAMP or by agents that activate adenylate cyclase. such as cholera toxin and forskolin (Fitz et al., 1982; Rodgers et al., 1983; Hoyer et al., 1984). Therefore. steroidogenesis in small cells appears to be regulated through a cAMP-mediated mechanism. The mechanisms regulating steroid production in large luteal cells are not clear. Prostsaglandins E_1. E_2 and I_2 enhance P_4 production by large cells (Fitz et al., 1984a.b) while dibutyryl cAMP, cholera toxin and forskolin are ineffective in stimulating P_4 production by large cells (Fitz et al.. 1982; Fitz et al., 1984a; Hoyer et al., 1984,1986). These results suggest a mechanism of signal

transduction which is not associated with adenylate cyclase.

LUTEOTROPISM

A luteotropin is a substance which can maintain the morphological and biochemical integrity of the CL. The luteotropin varies among species and may consist of a single hormone or a complex of hormones. For example, prolactin (PRL), FSH and LH are required for luteal maintenance in the hamster (Greenwald, 1967, 1973) and mouse (Choredary and Greenwald, 1969; Hilliard. 1973) while PRL, LH and estradiol are required in the rat (Hilliard, 1973). In most cases the luteotropin is of pituitary origin; however. estrogen is luteotropic in some species such as the rabbit (Keyes and Nalbandov, 1967) and rat (Hilliard, 1973). In the cow and ewe LH alone appears to be the major luteotropin. This conclusion is based upon a variety of studies utilizing in vivo and in vitro experimental models. In the cow and ewe corpora lutea either fail to form or regress following hypophysectomy at ovulation or mid-cycle, (Anderson et al., 1966; Kaltenbach et al., 1968a) and the effects of hypophysectomy can be reversed by the infusion of LH (Kaltenbach et al., 1968b). The in vivo administration of LH can overcome the luteolytic effects of oxytocin in the cow (Simmons and Hansel, 1964; Donaldson and Hansel. 1965b) and can prolong the lifespan of the CL (Wiltbank et al.. 1961; Donaldson and Hansel, 1965b; Seguin et al., 1977). In hysterectomized hiefers LH increased the total content and concentration of P_4 in the CL (Brunner et al.. 1969). while administration of antisera to LH resulted in premature regression of the CL in the cow (Snook et al., 1969) and ewe (Fuller and Hansel, 1970). In vitro, the addition of

purified LH enhances the production of P_4 (Mason. 1962; Savard et al.. 1965; Armstrong and Black. 1966; Selfart and Hansel. 1968), while the addition of LH antisera eliminates this response (Selfart and Hansel, 1968).

Although LH has received a considerable amount of attention as the primary luteotropin, an increasing body of literature has emerged concerning the role of other substances in the control of ovarian function. The following review of the literature concerns these other factors which may have a physiological role in the control of ovarian steroidogenesis.

FACTORS INFLUENCING OVARIAN FUNCTION

Catecholamines

The primary pathway catecholamines, norepinephrine (NE) and epinephrine (E), can stimulate the *in vitro* biosynthesis of cAMP and P_4 by luteal tissue from a variety of species: cow (Condon and Black, 1976; Godkin et al., 1977; Milvae and Hansel et al., 1983), rat (Harwood et al., 1979; Ratner et al., 1980; Norjavaara et al.. 1982), rabbit (Birnbaumer et al., 1976), ewe (Jordan et al., 1978), and sow (Perkins et al., 1986). In addition the alternative pathway catecholamines octopamine, synephrine and deoxyepinephrine can also enhance P_4 production by bovine luteal cells (Battista and Condon, 1986b). In contrast to the stimulatory effects of catecholamines in the aforementioned species, the CL of the mare (Condon et al., 1979) and human (Richardson and Masson, 1984) are refractory to stimulation by exogenous adrenergic agonists.

In vivo, the infusion of NE, E or the β-adrenergic agonist isoproternol (ISO) elevated plasma P_4 concentrations in the ewe (Bolt and Rollins, 1976), while infusion of NE increased cAMP levels in the CL of the rat (Norjavaara et al., 1983). In contrast, *in vivo* administration of NE and ISO was ineffective in stimulating ovarian steroidogenic activity in estrous rats, although fenoterol, a β_2-adrenergic agonist, resulted in enhanced P_4 production (Zsolnai et al., 1982). Infusion of E in early pregnant women increased plasma P_4 (Fylling, 1971a), but these effects may have been due to stimulation of placental P_4 production, rather than a direct effect on luteal function (Fylling, 1971b; Flint et al., 1974; Csapo and Herczeg, 1977). This possibility is supported by the finding that human luteal tissue is unresponsive to cateocholamines *in vitro*, as previously mentioned (Richardson and Masson, 1980; Casper and Cotterell, 1984). Infusion of ISO or the β-adrenergic antagonist propranolol in psuedopregnant rabbits did not alter luteal function (Gadsby et al., 1985). These authors suggested that endogenous catecholamines are not involved in the *in vivo* stimulation of luteal steroidogenesis. In the cow, administration of NE and E significantly elevated plasma P_4 concentrations, thus supporting a role for endogenous catecholamines in the control of bovine luteal function (Battista et al., 1987b).

Catecholamines act directly on luteal cells, since fibroblasts and erythrocytes separated from ewe luteal cell suspensions did not produce significant amounts of cAMP in response to E (Jordan et al., 1978). Adrenergic agents are thought to induce cAMP and subsequent P_4 production by binding to specific receptors in luteal cell membranes (Lefkowitz, 1976). Initial support for this hypothesis is suggested by

the finding that propranolol can block the catecholamine-induced increase in luteal cAMP and P_4 in the cow (Condon and Black, 1976; Godkin et al., 1978), rat (Harwood et al., 1980a; Norjavaara et al., 1982), and ewe (Jordan et al., 1978). These results not only suggest specific adrenergic receptors, but also that they are of the β-adrenergic type. Radioligand binding studies have demonstrated the presence of β-adrenergic receptors in rat luteal cell membranes (Coleman et al., 1978; Jordan, 1981; Kliachko and Zor, 1981). This receptor system has been further characterized to show that in the rat, the β-adrenergic stimulation of ovarian cAMP and P_4 is mediated by β_2-adrenergic receptors (Ratner et al., 1980; Adashi and Hsueh, 1981a; Laszlovszky and Erdo, 1983; Norjavaara et al., 1984). In contrast, the CL of the cow (Battista and Condon, 1986a) and sow (Perkins et al., 1986) contains a mixed population of β-adrenergic receptors.

Serotonin and Melatonin

Serotonin(5-hydroxytryptamine; 5-HT) has been detected in whole ovarian homogenates of the rat (Clausell and Soliman, 1978) and in bovine luteal tissue (Battista et al., 1987a), while ^{3}H-melatonin has been reported to be selectively taken up by the ovaries of the rat and cat *in vivo* (Wurtman et al., 1964). *In vitro* studies with human luteal tissue showed that melatonin stimulated P_4 production in a dose-dependent manner while 5-HT and N-acetyl 5-HT had no effect on P_4 production (MacPhee et al., 1975). In cultured rat granulosa cells, melatonin increased basal and LH-stimulated P_4 production (Fiske et al., 1984). In male rats melatonin and 5-HT inhibited the *in vitro* biosynthesis of androgens (Peat and Kinson, 1971; Ellis, 1972). In the cow, both 5-HT and 5-methoxytryptamine stimulate luteal P_4 secretion

while melatonin was ineffective in stimulating either basal or LH-stimulated P_4 production (Battista and Condon, 1986a; Battista et al., 1987a). Additionally, 5-HT has been shown to enhance bovine luteal P_4 production *in vivo* (Battista et al., 1987b).

Histamine and Dopamine

Although little is known about the role of histamine and dopamine in the control of ovarian function, Hirose et al. (1978) have reported that histamine stimulates cortisol and corticosterone production by dissociated adrenal cells of the dog. Greyard et al. (1983) found dopamine to inhibit the *in vitro* biosynthesis of androgens by rat testicular tissue. Utilizing bovine luteal tissue, Battista and Condon (1986a) were unable to demonstrate any stimulatory effect of histamine or a dopamine receptor agonist on basal or LH-stimulated P_4 production. However, we have recently found detectable levels of dopamine in bovine luteal tissue, and have found that dopamine stimulates P_4 production *in vitro* (Battista et al., unpublished observations).

Acetylcholine

As mentioned previously the mammalian ovary does receive cholinergic innervation, although the CL itself does not appear to contain cholinergic nerve fibers (Stefensen et al., 1981). That endogenous acetylcholine may play a role in influencing ovarian steroidogenesis is suggested by the findings that nicotinic, but not muscarinic agonists can inhibit FSH-induced P_4 production in cultured rat granulosa cells (Kasson and Hsueh, 1985) and hCG-stimulated androgen biosynthesis in cultured rat testicular cells (Kasson and Hsueh, 1985b). In contrast, neither acetylcholine nor synthetic cholinergic agonists altered basal or LH-induced P_4 production by human luteal tissue in short- or long-

term culture (Casper and Cotterell, 1984). Similarly, bovine luteal tissue is unresponsive to acetylcholine (Battista and Condon, 1986a) or synthetic nicotinic and muscarinic agonists (Battista and Condon, unpublished observations).

Gamma-Aminobutyric Acid

A possible role for γ-aminobutyric acid (GABA) in the control of ovarian function has been suggested due to the relatively high concentration of this compound in rat ovarian tissue (Del Rio and Caballero, 1980; Erdo et al., 1982; Schaeffer and Hsueh, 1982; Apud et al., 1984). In addition glutamate decarboxylase, the enzyme responsible for the synthesis of GABA has been identified in whole ovarian homogenates but not in enriched ovarian granulosa cells (Schaeffer and Hsueh, 1982; Apud et al., 1984). Specific, high affinity GABA binding sites have been demonstrated in the rat and guinea pig ovary (Erdo and Lapis, 1982; Schaeffer and Hsueh, 1982; Amenta et al., 1986) with a majority of those sites being associated with granulosa cells (Schaeffer and Hsueh, 1982). These receptors have been further characterized to be of the $GABA_A$ subtype (Amenta et al., 1986). In addition, a GABA-sensitive adenyl cyclase (Erdo and Lapis, 1982) and high affinity sodium uptake system (Erdo, 1983) have been demonstrated in the rat ovary. With respect to ovarian function, GABA acts to increase ovarian blood flow and to enhance follicular estrogen secretion (Erdo et al., 1985). The role of GABA in the regulation of luteal steroidogenesis is unclear, although incubation of bovine luteal cells with GABA did not alter basal or LH-stimulated P_4 production (Battista and Condon, 1986a).

Vasoactive Intestinal Peptide

Immunoreactive vasoactive intestinal peptide (VIP), has been

detected within the ovarian stroma of the mature (Larsson et al.. 1977; Alm et al., 1980a,b; Hakanson et al., 1982) and immature (Ahmed et al., 1986) rat ovary. VIP has been shown to stimulate. in a dose-dependent manner, the production of P_4 and estradiol in granulosa cells (Davoren and Hsueh, 1985) and whole ovarian homogenates (Ahmed et al., 1986) from the immature rat, and a role for VIP in the developmental regulation of ovarian steroidogenesis has been proposed (Ahmed et al., 1986). Intravenous infusion of VIP can elevate plasma P_4 concentrations in female rabbits (Fredricks et al., 1983), although this effect was later found to be due to VIP stimulation of adrenal P_4 secretion and this enhancement was independent of changes in LH and FSH (Fredricks et al., 1985). The role of VIP in the control of ovarian steroidogenesis in species other than the rat and rabbit is unknown.

Substance P

Nerve fibers containing substance P (SP) have been localized by immunofluorescence (Dees et al., 1985), radioimmunoassay and high performance liquid chromatography (Makris et al., 1982; Ojeda et al., 1985) in the immature rat ovary. The greatest amount of immunoreactive SP was associated with the ovarian vasculature. and no SP was detected in the CL (Dees et al., 1985). SP appears to be steroidogenically inactive since neither SP nor stable analogs altered basal or agonist-induced steroidogenesis in cultured granulosa cells or ovarian fragments obtained from immature rats (Ojeda et al.. 1985). Whether SP is present in ovarian tissues and can modulate steroidogenesis in other species is unknown.

Opiates

Immunoreactive β-endorphin has been localized in luteal tissue of

the rat (Tsong et al., 1982a; Lolait et al., 1985). mouse (Tsong et al.. 1982a; Shana et al., 1984), and ewe (Lim et al.. 1983), as well as in testicular cells of the rat and mouse (Tsong et al.. 1982a.b). The concurrent detection of larger molecular weight species of immunoreactive β-endorphin as well as the detection of ACTH. MSH and prooplomelanocortin (POMC) is suggestive of *de novo* synthesis (Tsong et al., 1982b; Lim et al., 1983; Shana et al., 1984; Lolait et al.. 1985). which appears to be regulated by gonadotropins (Shana et al., 1984; Lolait et al., 1985). A physiological role for opiate peptides in the control of luteal steroidogenesis has been suggested based on the observations of Abramowitz and Campbell (1983). who reported an enkephalin analog inhibited forskolin activation of adenylate cyclase in rabbit luteal membrane preparations. These observations suggest that the CL contains a receptor for opiate peptides which is coupled to an inhibitory guanine nucleotide component. and thus may serve as a negative autocrine regulator of luteal function (Abramowitz and Campbell, 1983). The role of opiate peptides in the control of steroidogenesis in other species has not been determined.

Oxytocin and Vasopressin

Oxytocin, oxytocin-associated neurophysin, and vasopressin have been observed in luteal tissue of the ewe (Wathes and Swann, 1982; Watkins 1983). cow (Fields et al., 1983; Wathes. 1983). pig (Wathes et al.. 1983; Pitzel et al., 1984). and human (Wathes et al., 1982; Khan-Dawood and Dawood, 1983). The CL appears to be responsible for the synthesis of these peptides (Wathes and Swann. 1982; Wathes et al., 1982. 1983), and recently the expression of the oxytocin-specific mRNA was reported in bovine luteal tissue (Ivell et al., 1985).

The physiological role of these intraluteal peptides is not clear, however oxytocin has been implicated in the processes of luteolysis (Fairdough et al., 1980; McCracken, 1980; Flint and Sheldrick, 1983) and ovulation (Virutamasen et al.. 1973, 1976).

With respect to regulating steroidogenesis, oxytocin and vasopressin have been reported to inhibit hCG-stimulated androgen synthesis in cultured rat Leydig cells (Adashi and Hsueh. 1981b). Tan et al. (1982a.b) have shown that high concentrations of oxytocin inhibited basal and hCG-enhanced P_4 production in bovine and human luteal cell preparations, while low concentrations of oxytocin enhanced basal steroidogenesis in both cell types. In contrast, oxytocin and vasopressin have no effect on basal or agonist-induced P_4 or cAMP production in luteal tissue of the rat (Mukhopadhyay et al., 1984) or human (Richardson and Masson. 1985). Similar results have been found for the effects of oxytocin in cultured bovine luteal cells (Burg, 1985).

Relaxin

The presence of relaxin has been demonstrated in luteal tissue of the cyclic and pregnant pig (Anderson et al.. 1973a; Sherwood and O'Byrne. 1974; Sherwood and Rutherford, 1981). rat (Anderson et al., 1973b; Anderson et al., 1975; Anderson and Long, 1978; Sherwood and Rutherford, 1981), human (O'Byrne et al., 1978; Quagliarello et al., 1980), and pregnant cow (Fields et al., 1980, 1982). It is generally believed that the CL is the principle and perhaps sole source of relaxin in the pig, rat and cow, and the specific mRNA for relaxin has been identified in the porcine CL (Gast et al., 1980; Gast. 1982). In the human, the placenta may also serve as a source of relaxin (Fields and

Larkin, 1981). Although the factors directing the synthesis and secretion of relaxin have not been well characterized, the concentration of relaxin in the CL and peripheral circulation is elevated during the latter stages of gestation (reviewed by Sherwood and Downing. 1983). A physiological role for relaxin in the control of myometrial activity (Downing and Sherwood, 1985b) and parturition (Downing and Sherwood, 1985a.c) has been shown. A role for relaxin in the control of ovarian steroidogenesis has not been demonstrated.

Insulin and Insulin-Like Growth Factors

Insulin and the insulin-like growth factor somatomedin-C, have in recent years received considerable attention as regulators of steroidogenesis. Insulin enhanced basal and gonadotropin-induced P_4 production in rat and bovine luteal tissue (Tesone et al., 1983; Ladenheim et al., 1984; Poff et al., 1987), porcine granulosa cells (Channing et al.. 1976; May et al., 1980; May and Schomberg, 1981; Veldhuis et al., 1983), and rat granulosa cells (Davoren and Hsueh, 1984; Adashi et al.. 1984. 1985a,b). Somatomedin-C has similar steroidogenic activity in that it can synergize with FSH to enhance P_4 production in rat granulosa cells (Adashi et al., 1984; 1985a,b). The action of insulin and somatomedin-C is independent of cAMP (Adashi et al., 1984; Veldhuis and Furlanetto, 1985), while the phosphorylation of plasma membrane proteins (D'Eroie et al.. 1984) and the induction of components of the cholesterol side-chain cleavage system (Veldhuis et al., 1986) have been associated with them. Additionally. both insulin and somatomedin-C enhance the induction of LH-receptors in rat (Adashi et al., 1985b) and porcine (May et al.. 1980; May and Schomberg. 1981) granulosa cells. These observations, plus the finding of discrete

binding sites for insulin and possibly for somatomedin-C (Hsueh et al., 1984; Ladenheim et al., 1984; Adashi et al., 1985a,b), and the observation that granulosa cells are capable of synthesizing somatomedin-like peptides (Hammond et al., 1983, 1984; Adashi et al., 1985b) suggest an autocrine role for insulin and insulin-like growth factors in the control of ovarian function.

Epidermal Growth Factor

Specific binding sites for epidermal growth factor (EGF) have been demonstrated in luteal, thecal and granulosa cells of the rat (Jones et al., 1982; Arnaud et al., 1983; Chabot et al., 1983) and cow (Vlodavsky et al., 1978). In the rat, EGF receptor concentration varies with the stage of the estrous cycle, with the highest concentration occuring at estrus (Arnaud et al., 1983). Additionally, EGF receptor synthesis was found to be regulated by gonadotropins (Arnaud et al., 1983).

With respect to steroidogenesis, EGF inhibited FSH-induced estrogen production in cultured rat granulosa cells (Hsueh et al., 1981; Welsh and Hsueh, 1982), and the production of P_4 in cultured porcine (Channing et al., 1983) and bovine (Savion and Gospodarowicz, 1980; Franchimont et al., 1986) granulosa cells. In contrast, EGF enhanced basal and agonist-induced P_4 production in granulosa cells of the rat (Jones et al., 1982; Knecht and Catt, 1983). The divergent effects of EGF in regulation of rat granulosa cell steroidogenesis is associated with EGF inhibition of aromatase activity and stimulation of 3β-hydroxysteroid dehydrogenase activity (Jones et al., 1982).

Gonadotropin-Releasing Hormone

Treatment with gonadotropin-releasing hormone (GnRH) or its agonists *in vivo* results in paradoxical inhibition of female reproductive

function (Corbin et al., 1978; Nillius et al., 1978; Rivier et al.. 1978). *In vitro*, GnRH or its analogues inhibited gonadotropin-induced cAMP and steroidogenesis in rat granulosa (Hsueh and Erickson. 1979; Hsueh and Ling, 1979; Jones and Hsueh. 1981) and luteal cells (Clayton et al., 1979; Behrman et al., 1980; Harwood et al., 1980b). Furthermore, specific GnRH receptor sites have been identified in rat follicular and luteal tissue (Clayton et al., 1979; Harwood et al.. 1980b; Pieper et al., 1981). In addition, the inhibitory effects of GnRH on granulosa cells involved the activation of a plasma membrane cAMP phosphodiesterase (Knecht and Catt, 1981). Treatment of rat granulosa cells with GnRH alone enhanced cAMP and progestin production (Knecht et al.. 1981; Clark et al.. 1982; Jones and Hsueh, 1982a,b; Ranta et al.,1982). although these effects were not observed in luteal cells (Behrman et al., 1980; Harwood et al., 1980).

In contrast to the effects of GnRH on steroidogenesis in the rat, no direct effects of GnRH have been observed in ovarian tissues of the human (Tan and Biggs, 1983), monkey (Asch et al., 1981a). or cow (Kuman. 1984). In addition no GnRH binding was observed in ovarian tissues of the human (Clayton. 1982) or monkey (Asch et al., 1981b).

Adenosine

Adenosine and other adenine-derived purines can amplify LH-dependent cAMP accumulation and P_4 production in rat (Hall et al., 1981; Behrman et al., 1982; Brennan et al.. 1983) and human (Polan et al., 1983) luteal tissue. Adenosine can also enhance FSH-dependent cAMP and P_4 production in human and rat granulosa cells (Polan et al., 1983). The stimulatory effects of adenosine have also been shown in rat Leydig and adrenal cells (Wolff and Hope-Cook, 1977; Rommerts et al.. 1984).

Moreover, the antigonadotropic effect of $PGF_{2\alpha}$ on luteal cAMP and P_4 production was completely antagonized by adenosine (Behrman et al., 1982).

The amplification of gonadotropin-induced cAMP accumulation by adenosine is dependent upon an intracellular site of action since the effects of adenosine can be blocked by dipyridamol, an inhibitor of nucleoside transport (Hall et al., 1981; Brennan et al., 1983), and a selective transport system for adenosine has been demonstrated in rat luteal cells (Behrman et al., 1983). The mechanism underlying adenosine amplification of LH-dependent cAMP accumulation is due to utilization of extracellular adenosine as a substrate for ATP formation, and thus acts as a prosubstrate for conversion into cAMP by adenylate cyclase (Behrman et al., 1983; Brennan et al., 1983). Whether an adenosine uptake mechanism is functional in bovine luteal cells is at this time unknown.

Prostaglandins

The bovine CL can synthesize prostaglandins, and this synthesis can be stimulated by LH (Shemesh and Hansel, 1975a,b; Hixon and Hansel, 1975; Milvae and Hansel, 1983) and inhibited by indomethacin (Milvae and Hansel, 1985). Rat granulosa cells can also synthesize prostaglandins *in vitro*, and LH can act to increase prostaglandin synthetase activity (Clark et al., 1978; Koos and Clark, 1982). Prostaglandin receptors have been identified in a variety of species including the cow (Kimball and Lauderdale, 1975; Rao, 1974,1975), ewe (Powell et al., 1974a), rabbit (Abramowitz and Birnbaumer, 1979), and human (Powell et al., 1974b).

Prostglandins of the E-series (PGE_1 and PGE_2) and I-series (PGI_2) interact with luteal membrane receptors to enhance cAMP and P_4

production (Marsh, 1971; Abramowitz and Birnbaumer, 1979; Milvae and Hansel, 1980, 1983). While prostaglandins of the F-series (noteably PGF_{2}^{α}) act to inhibit luteal steroidogenesis *in vitro* (for review of literature see Pate and Condon, 1984) and *in vivo* (reviewed by Inskeep and Murdoch, 1980) and a physiological role for PGF_{2}^{α} in luteolysis (McCracken et al., 1972; McCracken, 1980) and ovulation (reviewed by Espey. 1978) has been proposed.

Glucocorticoids

Administration of pharmacological doses of glucocorticoids blocked ovulation in women and laboratory animals (Hagino. 1972). Treatment of rat granulosa cells with cortisol, inhibited FSH-induced estrogen production (Hsueh and Erickson, 1978), while stimulating the production of P_4 (Adashi et al., 1981). The divergent effects of glucocorticoids on rat granulosa cell steroidogenesis is associated with inhibition of aromatase activity and stimulation of 3β-hydroxysteroid dehydrogenase activity (Adashi et al., 1981). In male rats, *in vivo* treatment with glucocorticoids decreased plasma testosterone concentrations (Saez et al., 1977; Bambino and Hsueh, 1976, 1979). Incubation of cultured rat Leydig cells with glucocorticoids inhibited basal and LH-induced androgen production and reduced LH-receptor synthesis (Bambino and Hsueh, 1981).

That glucocorticoids may be of physiological significance in the control of steroidogenesis is suggested by the presence of receptors in rat granulosa cells (Lorevel et al.. 1977) and in rat testicular tissue (Ballard et al., 1974; Evain et al., 1976). Data concerning the effects of glucocorticoid treatment on luteal steroidogenesis is not available.

Catecholestrogens

The catecholestrogens 2- and 4-hydroxyestradiol are naturally occuring compounds formed from estradiol by the enzyme estrogen-2/4-hydroxylase, and are considered to be the major biproducts of estradiol metabolism in mammals (reviewed by Ball and Knuppen, 1980). Catecholestrogens can mimic or alter estrogen responses through their interaction with estrogen receptors (MacLusky et al., 1983), and can influence enzymes regulating the biosynthesis and catabolism of catecholamines (Lloyd and Welsz, 1978).

The role of estradiol in the enhancement of gonadotropin-induced steroidogenesis and receptor induction in rat ovarian tissue is well established (reviewed by Hsueh et al., 1984). Recently, catecolestrogens have been shown to enhance steroidogenesis in rat granulosa (Hudson and Hillier, 1985) and luteal cells (Khan and Gibori, 1984). These results, plus the observation of catecholestrogen formation in rat (Barbieri et al., 1978) and porcine (Hammond et al., 1986) follicular tissue, suggest that catecholestrogens are intraovarian regulators of steroidogenesis.

Despite the existence of estrogen receptors in rat follicular and luteal tissue (Richards, 1974), it does not appear that catecholestrogens are acting as weak estrogens (Hudson and Hillier, 1985; Hammond et al., 1986). Recently Barnea and Fakih (1985) reported that catecholestrogens stimulate placental steroidogenesis, and that their effects were mediated by α and β-adrenergic receptors. Thus, it is possible that catecholestrogens may enhance steroidogenesis through interactions with β-adrenergic receptors, which are present on rat ovarian cells (Coleman et al., 1979). Both the cow and ewe have luteal

estradiol receptors (Kimball and Hansel, 1974; Glass et al., 1984) as well as β-adrenergic receptors (Condon and Black, 1976; Jordan et al., 1978). However , estradiol is luteolytic in both of these species (reviewed by Hansel, 1973; Williams and Marsh, 1978). The possibility that catecholestrogens may enhance luteal steroidogenesis in the cow and ewe through interaction with β-adrenergic receptors has not been investigated.

MECHANISMS CONTROLLING STEROIDOGENESIS

Hormone-Receptor Interaction: Activation of Adenylate Cyclase

Initial events mediating steroidogenesis involve the interaction of a hormone with a specific membrane receptor. As discussed in the previous section, luteal cells (and/or granulosa cells) contain a variety of receptors which mediate both stimulatory and inhibitory control on steroidogenesis (reviewed by Hsueh et al., 1983;1984). Hormone-receptor interaction results in the formation of a hormone-receptor complex (H-R complex) which interacts with a regulatory protein which then activates adenylate cyclase, a membrane-associated enzyme which catalyzes the conversion of MgATP to cAMP (Marsh, 1970). Other membrane-associated events which are coupled to H-R interaction include membrane methylation, which results in alteration of membrane fluidity (Hirata et al., 1979; Miivae et al., 1983). The decrease in membrane microviscosity acts to increase the lateral mobility of the H-R complex, thus enhancing coupling to adenylate cyclase and unmasking membrane receptors (reviewed by Hirata and Axelrod, 1980; Axelrod and Hirata, 1982). Additionally, H-R interaction is associated with clustering and

internalization of membrane receptors (reviewed by Willingham and Pastan, 1984), which are either packaged into vesicles for degradation (Ahmed et al., 1981) or remain biologically active and further alter intracellular responses (Zimniski et al.. 1982; Rao et al., 1983).

The hormone-sensitive adenylate cyclase system is composed of three membrane bound components; a receptor which has a high affinity and specificity for the ligand, a guanine nucleotide regulatory protein or G-protein (N), and a catalytic unit (reviewed by Rodbell, 1980; Birnbaumer and Kirchick, 1983). The regulatory unit is a dimeric protein composed of two subunits designated N_α and N_β. The N_α contains the binding sites for GTP and the catalytic unit, while N_β contains a Mg ion binding site and the ability to interact with the H-R complex (reviewed by Hildebrand et al., 1984a). Upon binding of the hormone to its receptor the H-R complex couples with N_β and causes a shift in the K_m of N for Mg from 10mM to less than 0.5mM, which is the physiological concentration of Mg. Binding of Mg to the N_β portion induces dissociation of the N protein allowing N_α to interact with and activate the catalytic unit. There is also a requirement for the nucleotide GTP, which when bound to N_α allows it to couple with the catalytic unit. The activated catalytic unit is then able to enhance the conversion of MgATP to cAMP. Therefore. hormones act primarily by reducing the Mg requirement for GTP-dependent activation of adenylate cyclase. Return of the system to the nonstimulated state is dependent upon the GTPase activity associated with N_α. Thus hydrolysis of GTP reverses the binding of N_α to the catalytic unit resulting in inactivation of adenylate cyclase (reviewed by Hildebrant et al., 1984; Birnbaumer et al., 1985a,b). The inhibitory effects of various hormones and

neurotransmitters upon adenylate cyclase and subsequent cAMP formation are mediated by a distinct GTP regulatory protein designated N_I (Jakobs, 1979; Cooper. 1982; Gilman, 1984). N_I. like N_S, undergoes subunit dissociation with subsequent binding to and inactivation of the catalytic unit. The exact mechanism whereby N_I attenuates the activity of the catalytic unit is unknown (reviewed by Rodbell, 1980; Birnbaumer et al.. 1985a.b).

cAMP and cAMP-Dependent Protein Kinase

Addition of cAMP to luteal tissue increased P_4 production while incubation with LH resulted in a rapid accumulation of cAMP prior to P_4 synthesis. Subsequently it was determined that the action of LH was mediated by adenylate cyclase rather than the inhibition of phosphodiesterase activity (reviewed by Marsh, 1976; Williams et al.. 1978). The biological effects of cAMP are mediated through a specific cAMP-dependent protein kinase (PK_A), the activation of which involved agonist-induced steroidogenesis (Menon, 1973; Vaitukaitis et al., 1975; Azhar et al., 1976; Ling and Marsh, 1977). PK_A is composed of regulatory and catalytic subunits, which in the inactive state exist as a holoenzyme. Binding of two molecules of cAMP to the regulatory units induces subunit dissociation and release of two enzymatically active catalytic subunits (reviewed by Walsh and Ashley, 1973; Krebs and Beavo. 1979). The active catalytic subunit can then phosphorylate a variety of intracellular proteins, and a correlation in protein phosphorylation and P_4 synthesis has been observed in luteal tissue (Darbon et al., 1981).

Regulation of Steroidogenesis: Protein Kinase A

There are three sources of cholesterol which can be utilized for the synthesis of P_4: de novo synthesis from acetate, cholesterol derived

from circulating lipoproteins, or cholesterol derived from stored cholesterol esters (reviewed by Dorrington, 1977). The de novo synthesis of cholesterol is regulated by the enzyme 3-hydroxy-3-methylglutaryl-coenzyme A reductase (Brown et al., 1973), which is regulated by a phosphorylation-dephosphorylation mechanism (Beg et al., 1980; Ingebritsen et al., 1981), the activity of which is induced by LH (Kovanen et al., 1978; Schuler et al., 1981). PK_A can also phosphorylate and activate cholesterol ester hydrolase resulting in the hydrolysis of cholesterol esters to yield free cholesterol (Trzeciak and Boyd, 1973; Beckett and Boyd, 1977; Cooke et al., 1983; Colbran et al., 1986). Intracellular cholesterol must then be transported to the mitochondria where it is converted to pregnenolone in a rate limiting reaction catalyzed by the cholesterol side-chain cleavage enzyme system (reviewed by Savard, 1973). The side-chain cleavage system resides in or on the inner mitochondrial membrane and is composed of three proteins: 1) luteodoxin reductase, an FAD-containing flavoprotein, 2) cytochrome P_{450scc}, the terminal oxygenase, and 3) luteodoxin, which serves to shuttle electrons between the reductase and cP_{450} (reviewed by Sulimovici and Boyd, 1969). The cholesterol side-chain cleavage reaction involves two sequential hydroxylations of cholesterol at carbon 22 and carbon 20 followed by cleavage of the cholesterol side-chain between carbon 20 and 22. Each step in the reaction requires oxygen and NADPH, thus the conversion of one mole of cholesterol to one mole of pregnenolone requires three moles of oxygen and NADPH (reviewed by Burstein and Gut, 1971).

Many investigators have demonstrated that agonist-induced steroidogenesis is dependent upon protein synthesis (reviewed by Garren

et al., 1971; Savard et al., 1976). Although it has been suggested that PK_A acts directly on the side-chain cleavage system (Caron et al., 1975; Williams et al.,1978), recent reports have shown that PK_A has no direct regulatory action on mitochondrial steroidogenesis (Downing and Dimino. 1979; Inaba and Wiest, 1985). The observation that the transport of cholesterol to the inner mitochondrial membrane is translation dependent (Crivello and Jefcoate. 1980; Mori and Marsh, 1982), coupled with the finding that PK_A is translocated to the nucleus where it may regulate transcription (Jungmann et al., 1974; Spielvogel et al., 1977), lead to the proposal that stimulation of steroidogenesis is dependent upon the synthesis of a labile regulatory protein (Simpson et al.. 1978). The labile protein is thought to be generated on cytoplasmic ribosomes. since chloramphenicol. an inhibitor of mitochondrial protein synthesis. was ineffective in blocking steroidogenesis (Arthur and Boyd. 1974). The labile protein may enhance agonist-induced steroidogenesis by aiding in the transport of cholesterol across the mitochondrial membrane, thereby enhanceing cholesterol side-chain cleavage activity (reviewed by Strauss et al., 1981).

After the conversion of cholesterol to pregnenolone. pregnenolone diffuses out of the mitochondria to the smooth endoplasmic reticulum where it is converted to P_4 by the enzymes Δ^5-3β-hydroxysteroid dehydrogenase (3β-HSD) and Δ^5 Δ^4-isomerase (Tamaoki. 1973). The enzyme 20α-hydroxysteroid dehydrogenase (20α-HSD) is also located in the smooth endoplasmic reticulum and acts to convert P_4 to the less potent progestin 20β-dihydroprogesterone (Tamaoki, 1973). With respect to the control of steroidogenesis. both 3β-HSD and 20α-HSD are under hormonal regulation in rat granulosa cells. Stimulatory agents such as LH, FSH

catecholamines increase the enzymatic activity of 3β-HSD while decreasing 20α-HSD activity. Inhibitory substances, such as GnRH, act to regulate these enzymes in a reverse fashion. The mechanism whereby these enzymes are regulated in other species is unknown (reviewed by Hsueh et al., 1983, 1984).

Regulation of Steroidogenesis: Protein Kinase C

Protein kinase C (PK_C) is a cytosolic enzyme composed of a single polypeptide chain. The enzyme does not appear to consist of subunits since activation is not associated with dissociation of the enzyme molecule (reviewed by Kuo et al., 1984). Activation of PK_C is dependent upon phosphotidylinositol (PI) hydrolysis and intracellular Ca^{2+} (reviewed by Kuo et al., 1984; Nishizuka, 1984). PI comprises less than ten percent of the total phospholipid in mammalian membranes. most of it associated with the plasma membrane although some is associated with the mitochondria and the endoplasmic reticulum (reviewed by Mitchell, 1975). Hydrolysis of PI is associated with agonist-induced phosphorylation of PI by specific kinases to form phosphatidylinositol 4-phosphate (PIP) and phosphatidylinositol 4,5-bisphosphate (PIP_2). PIP_2 is acted upon by phospholipase-C to yield diacylglycerol (DAG) and inositol triphosphate (IP_3). IP_3 acts to enhance the intracellular mobilization of Ca^{2+}, thus promoting the production of DAG by the Ca^{2+}-dependent phospholipase-C (reviewed by Farese, 1983a,b, 1984). DAG. in conjunction with Ca^{2+}. activates PK_C by increasing its affinity for Ca^{2+}. The activated PK_C then acts synergistically with mobilized Ca^{2+} to induce cellular responses. Thus. PK_C is a Ca^{2+}-dependent. phospholipid-sensitive enzyme, which when activated acts in a synergistic manner with intracellular Ca^{2+} to elicite a full physiological response (reviewed by

Kuo et al., 1984; Nishizuka, 1984).

With respect to steroidogenesis, LH has been shown to increase PI hydrolysis in bovine luteal tissue (Davis et al., 1981) and rat granulosa cells (Davis et al., 1983). PK_C activity has been demonstrated in cytosolic preparations from the bovine CL (Davis and Clark, 1983) and human ovary (Clark et al., 1983). Use of phorbol esters, which can substitute for DAG and directly activate PK_C (Castagna et al., 1982; Niedel et al., 1983), have been shown to stimulate steroidogenesis in bovine luteal tissue (Brunswig et al., 1986) and rat granulosa cells (Kawai and Clark, 1985; Shinohara et al., 1986). The mechanism whereby PK_C mediates agonist-induced steroidogenesis is unknown.

Progesterone Secretion

Once synthesized , P_4 must be secreted from the CL into the bloodstream. It has been generally accepted over the years that the synthesis of steroid hormones is directly coupled to stimulation, and that newly synthesized steroids leave the cells within minutes by passive diffusion (Vogt, 1943; Short, 1964; Enders, 1973; Baird, 1977; Rice et al., 1986). However, some data suggest that the secretion of P_4 may be an active process. For example, P_4 secretion requires Ca^{2+} (Higuchi et al., 1976), and K^+-induced membrane depolarization is associated with a rapid enhancement in P_4 secretion (Higuchi et al., 1976). It has been suggested that P_4 binds to cytosolic proteins and is packaged into granules which are released by an exocytotic mechanism (Gemmell et al., 1974; Quirk et al., 1979; Sawyer et al., 1979). Two specific P_4 binding proteins have been found in cytosolic preparations of bovine luteal tissue (Willcox, 1983). However, alteration of

cytoskeletal structures important in cellular transport were found to have no effect on P_4 secretion (Zor et al., 1978; Azhar and Menon, 1981; Gwynne and Condon, 1982). Additionally, Rice et al. (1986) were unable to demonstrate the presence of P_4 in secretory granules within the ovine corpus luteum. Furthermore, evidence suggests that instead of secretory granules, serum steroid binding proteins may facilitate the transport of steroid hormones from their site of synthesis into the blood stream (Condon and Pate, 1981).

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EXPERIMENT ONE

Serotonin-induced stimulation of progesterone production by cow luteal cells in vitro

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Summary

The addition of acetylcholine or histamine (10^{-7} to 10^{-4}M), γ-aminobutyric acid, a dopamine agonist, and melatonin (10^{-7} to 10^{-5}M) did not alter basal or LH-stimulated progesterone production ($P>0.05$). The addition of the specific β_2-adrenergic agonists terbutaline and salbutamol did not significantly elevate progesterone production. Treatment of luteal cells with serotonin (5-HT), 10^{-6} to 10^{-4}M increased the production of progesterone ($P<0.05$). This stimulated production was inhibited by the addition of mianserin (10^{-5}M, a 5-HT antagonist; $P<0.05$). Isoproterenol (10^{-7} to 10^{-4}M) also resulted in significant increases in progesterone production ($P<0.05$). The combined treatments of 5-HT + LH, isoproterenol + LH, or isoproterenol + 5-HT did not result in a further increase in progesterone above that observed in response to LH or isoproterenol alone ($P>0.05$). The isoproterenol-induced progesterone production could not be blocked by butoxamine (10^{-5}M, a β_2-antagonist), or practolol (10^{-5}M, a β_1-antagonist), but was inhibited by propranolol (10^{-5}M, a general β-antagonist; $P<0.05$). The response to isoproterenol was uneffected by mianserin (10^{-5}M). These results demonstrate apossible role for 5-HT in the stimulation of steroidogenesis by the bovine corpus luteum. Furthermore, these results suggest that the 5-HT-induced progesterone production is a receptor-mediated event.

Introduction

At the level of the ovary the adrenergic agents adrenaline, noradrenaline and isoproterenol have been shown to stimulate cAMP and progesterone production by luteal tissue from the cow (Condon & Black, 1976; Godkin, Black & Duby, 1977; Milvae, Alila & Hansel, 1983), rat (Harwood, Richert, Dufau & Catt, 1980; Ratner, Sanborn & Weiss, 1980; Norjavaara, Selstam & Ahren, 1982), rabbit (Birnbaumer, Yang, Hunzicker-Dunn, Brockaert & Duran, 1976), and ewe (Jordan, Caffrey & Niswender, 1978). In contrast to the stimulatory effects of catecholamines in the aforementioned species, corpora lutea of the mare (Condon, Ganjam & Kenney, 1979) and woman (Richardson & Masson, 1980; Casper & Cotterell, 1984) appear to be refractory to stimulation by exogenous adrenergic agonists.

Catecholamines act directly on luteal cells, since fibroblasts and erythrocytes separated from ewe luteal cell suspensions did not produce significant amounts of cAMP in response to adrenaline (Jordan et al., 1978). Adrenergic agents are thought to induce cAMP and subsequent progesterone production by binding to specific receptors in the luteal cell membrane (Lefkowitz, 1976). Initial support for this hypothesis is suggested by the finding that propranolol can block the catecholamine-induced increase in luteal cAMP and progesterone production in the cow (Condon & Black, 1976; Godkin et al., 1977), rat (Harwood et al., 1980; Norjavaara et al., 1982) and ewe (Jordan et al., 1978). These results not only suggest specific adrenergic receptors, but also that they are of the β-adrenergic type. In the rat the β-adrenergic stimulation of ovarian cAMP and progesterone has been shown to be mediated by β_2-

adrenergic receptors (Coleman, Paterson & Somerville, 1979; Ratner, Weiss & Sanborn, 1980; Adashi & Hsueh, 1981; Norjavaara, Roseberg, Gafvels & Selstam, 1984). In the cow and ewe the characterization of a specific β_2-adrenergic mediation of catecholamine response by luteal cells has not been established. The purpose of this study with cow luteal cells was to determine (a) whether the catecholamine-induced increase in progesterone production is mediated by a β_2-adrenergic receptor and (b) the role of other biogenic amines on basal and LH-stimulated progesterone production.

Materials and Methods

Materials and drug preparation

Serotonin (5-hydroxytryptamine oxalate salt; 5-HT), dl-isoproterenol HCl, acetycholine chloride, histamine dihydrochloride, salbutamol, γ-amino-n-butyric acid (GABA), melatonin (N-acetyl-5-methoxytryptamine) and dl-propranolol HCl were purchased from Sigma Chemical Co., St. Louis, MO. Mianserin HCl and 2-amino-6,7-dihydroxy-1,2,3,4-tetrahydronaphthalene hydrobromide were from Research Biochemicals, Inc., Wayland, MA, and butoxamine HCl was from Burrough's Wellcome Co., Research Triangle Park, NC. Terbutaline was donated by Ciba-Geigy Corp., Summit, NJ, practolol was donated by Imperial Chemical Industries PLC, Macclesfield, Cheshire, U.K. Luteinizing hormone (LH) was supplied by the National Institutes of Health (NIAMDD-bLH-4). Ham's F12 culture medium was purchased from Grand Island Biological Co., Grand Island, NY. The collagenase was Worthington, Type 1, Freehold, NJ, and the N-2-hydroxythylpiperazine-N-2-ethanesulphonic acid (Hepes) was purchased from Calbiochem-Behring, San Diego, CA.

Salbutamol and terbutaline are specific β_2-adrenergic receptor agonists, while isoproterenol is a nonspecific β-agonist, activating both subtypes of β-receptors. Butoxamine is a specific β_2-adrenergic receptor antagonist and practolol a specific β_1-antagonist; propranolol is a nonspecific β-adrenergic blocking agent. Mianserin is a nonspecific serotonin receptor antagonist which blocks both 5-HT_1 and 5-HT_2 receptor subtypes and 2-amino-6,7-dihydroxy-1,2,3,4-tetrahydronapthalene hydrobromide is a nonspecific dopamine agonist. All hormone and drug preparations were prepared in cold 0.05M phosphate-

buffered saline (pH 7.4) before use in each experiment and used at a final concentration of 10^{-7} to 10^{-4}M. In all cases N equals the number of animals tested. Duplicate determinations of all treatments were performed for each corpus luteum.

Experimental procedures

Corpora lutea (CL) were obtained per vaginam from regularly cycling, nonlactating dairy cows on days 10-12 of the oestrous cycle (oestrus=Day 0). The luteal tissue was placed into Ham's F12 culture medium containing 24 mM Hepes (pH 7.4) at 4°C for transport back to the laboratory. The luteal tissue was dissociated using collagenase (2000 U/g tissue) as described by Simmons, Caffrey, Phillips, Abel & Niswender (1976) and modified by Pate & Condon (1982). Cell viability was determined by the trypan blue exclusion method (Tennant, 1964). Incubations were conducted utilizing 2.5×10^5 viable cells/culture tube in a final volume of 1 ml of Ham's F12 culture medium. Luteal cell suspensions were incubated in a Dubnoff metabolic shaker water bath at 37°C starting with a 15 min preincubation period, after which the cells were centrifuged (800 g, 10 min at 4°C) and the medium discarded. The cells were then resuspended in fresh medium and the appropriate treatment solutions added. After the incubation period of 2-h, cell suspensions were centrifuged and the culture medium was collected and stored (-20°C) for subsequent determination of progesterone concentration.

Progesterone present in the culture medium was quantitated by radioimmunoassay of unextracted samples using antiprogesterone-11-bovine serum albumin (No. 337, Niswender). This antiserum does not cross-react

significantly with any other steroid present in the incubation medium (Gibori, Antczak, & Rothchild, 1977). The progesterone tracer used was [1,2-^{3}H] progesterone and was purchased from New England Nuclear (Boston, MA). The sensitivity of the assay as determined by the lower 95% confidence limit of the maximum binding in the absence of any unlabeled progesterone was 0.1 ng. The intraassay and interassay coefficients of variability were 5.3% and 13.3%, respectively. All standards were assayed in quadruplicate, and all unknowns were assayed in duplicate.

Statistical analysis

Analysis of differences between treatment means was conducted using one-way analysis of variance followed by the Student-Newman-Keuls mean separation procedure.

Results

The effects of some of the biogenic amines on basal progesterone production are presented in Table 1. Progesterone production was not affected significantly by acetylcholine, histamine, GABA, dopamine or melatonin over the treatment concentrations tested. Similarly, none of these compounds had any effect on LH-stimulated progesterone production ($P>0.05$, Table 2). Terbutaline and salbutamol did not affect the basal or LH-stimulated production of progesterone. LH (100 ng/ml) alone resulted in a large increase in progesterone production ($P<0.01$).

The addition of serotonin at concentrations from 10^{-6} to 10^{-4}M stimulated the production of progesterone by luteal cells ($P<0.05$, Text-fig. 1). Additionally, the 5-HT induced progesterone response could be blocked by the addition of mianserin (10^{-5}M, $P<0.05$). The 5-HT stimulation of progesterone production was significantly less than that observed for LH alone and there was no additive effect of 5-HT combined with LH. Isoproterenol, at concentrations from 10^{-7} to 10^{-4}M, also resulted in stimulation of progesterone production ($P<0.05$, Text-fig. 2). The combination of isoproterenol plus LH or isoproterenol plus 5-HT (10^{-5}M) did not result in a significant elevation of progesterone above that observed with LH or isoproterenol, respectively ($P>0.05$). The effect of various β-adrenergic receptor antagonists and mianserin upon isoproterenol- stimulated progesterone is presented in Text-fig. 3. The isoproterenol- induced response could not be inhibited by butoxamine (10^{-5}M) or practolol (10^{-5}M) at any of the isoproterenol concentrations tested ($P>0.05$). Similarly, mianserin (10^{-5}M) did not block progesterone production by isoproterenol ($P>0.05$). In contrast,

propranolol (10^{-5}M) did inhibit the isoproterenol-induced response at an isoproterenol concentration of 10^{-7} to 10^{-5}M ($P<0.05$). None of the antagonists used in these studies significantly affected either basal or LH-stimulated progesterone production (data not shown).

Discussion

These results demonstrate a possible role for serotonin in the stimulation of steroidogenesis by the bovine CL. That the serotonin-induced stimulation of progesterone production could be inhibited by a serotonin receptor antagonist suggests its action to be a receptor-mediated event. A preliminary experiment with C. E. Rexroad, Jr. (USDA, Beltsville) has shown serotonin to be present within the bovine CL at a relatively high concentration (152.7$\pm$40.19 ng/g). In the pineal gland of the cow, serotonin concentrations range from 1000 to 2000 ng/g depending upon the time of year (Philo & Reiter, 1980). In the rat, Clausell & Soliman (1978) have reported 5-HT to be present in ovarian tissue: the concentration of 5-HT was approximately 900 ng/g during both dioestrus and metoestrus with a significant increase to 1300 ng/g occurring at oestrus and a role for ovarian 5-HT in the process of ovulation was proposed.

Possible sources of luteal 5-HT include mast cells, blood platelets and de novo synthesis. In this laboratory, histological determination of mast cells using Giemsa, toluidene blue and methylene blue staining techniques has not conclusively demonstrated their presence in luteal tissue of cows. It is possible that serotonin may be working indirectly to stimulate progesterone production by acting on a secondary cell type such as endothelial cells to produce a luteotrophin. Studies are presently underway to evaluate this possibility and to further define the role of serotonin and its' metabolites inthe stimulation of steroidogenesis by the bovine CL.

<u>in vitro</u> studies using human CL slices showed that melatonin

stimulated progesterone production in a dose-dependent manner while serotonin and N-acetyl serotonin had no effect on progesterone production (MacPhee, Cole, & Rice, 1975). In cultured rat granulosa cells, melatonin has been reported to increase basal and LH-stimulated progesterone production (Fiske, Parker, Ulmer, Ow & Aziz, 1984). [^{3}H]-melatonin has been reported to be selectively taken up by the ovaries in the rat and cat *in vivo* (Wurtman, Axelrod & Potter, 1964). In male rats melatonin and serotonin have been demonstrated to inhibit the *in vitro* biosynthesis of androgens (Peat & Kinson, 1971, Ellis, 1972). In our studies utilizing dissociated bovine luteal cells melatonin was unable to alter either basal or LH-stimulated progesterone production.

A possible role for GABA in the regulation of ovarian function has been suggested due to the relatively high concentration of this coumpound in rat ovarian tissue (Del Rio & Caballero, 1980). Shaeffer & Hsueh (1982) have reported GABA and glutamate decarboxylase (GAD), the enzyme responsible for GABA synthesis, to be present in whole ovarian homogenates but not in enriched ovarian granulosa cells. Additionally, these authors have demonstrated specific binding sites for GABA in the rat ovary, with the majority of these sites being associated with granulosa cells. The testing of GABA in our short-term incubation system does not support a role for GABA in the control of steroidogenesis by the bovine CL.

To our knowledge, there is only a single report in the literature concerning the effects of acetylcholine on luteal function. In that study, acetylcholine and cabachol (a synthetic cholinergic agent) at concentrations up to 10^{-4}M did not effect basal or LH-stimulated progesterone production by human luteal tissue in either a short-term (4

h) or long-term (10 day) culture system (Casper & Cotterell, 1984). There are no data concerning the effects of histamine and dopamine on luteal steroidogenesis. However, histamine has been reported to induce the production of cortisol and corticosterone by dissociated adrenal cells of the dog (Hirose, Matsumato & Aikawa, 1978). In our studies we have been unable to demonstrate any significant influence of acetylcholine, histamine or dopamine in controlling luteal steroidogenesis.

The isoproterenol stimulation of progesterone reported in this study is consistent with previous results reported for bovine luteal slices (Condon & Black; 1976, Godkin et al., 1977) and dissociated bovine luteal cells (Milvae et al., 1983). Neither terbutaline nor salbutamol, the two β_2-adrenergic agonists used in the present study, significantly affected progesterone production by bovine luteal cells. These results differ from those reproted for rat luteal tissue (Ratner et al., 1980; Norjavaara et al., 1984) and FSH-primed rat granulosa cells (Adashi & Hsueh, 1981). Additionally, isoproterenol was reported to stimulate cAMP by rat luteal tissue, and this response could not be blocked by a β_1- or a β_2-adrenergic receptor antagonist, although it could be blocked by propranolol. Similar results have been found in this study for progesterone production by bovine luteal cells. The isoproterenol stimulation of progesterone production could not be blocked by butoxamine or practolol, but was inhibited by propranolol. The effects of isoproterenol either alone or in combination with these antagonists, on progesterone production by rat luteal tissue was not reported by Ratner et al. (1980). It may be that the β-adrenergic agonists used in these studies lack the specificity and affinity for β-adrenergic

receptors on bovine luteal cell membranes. In the rat, salbutamol and terbutaline have been shown to be partial agonists and their affinity varies between tissue types (Coleman et al., 1979; Adashi & Hsueh, 1981; Laszlovszky & Erdo, 1983; Norjavaara et al., 1984). As suggested by Coleman, Paterson & Somerville (1979) it is also possible that these β-adrenergic agonists and antagonists are metabolized more readily than isoproterenol or propranolol thus reducing their biological activity. Since neither, β_1-, nor β_2-adrenergic antagonists blocked the isoproterenol stimulation of progesterone but propranolol, an adrenergic antagonist which blocks both receptor subtypes, was able to, these results may suggest a mixed population of β-adrenergic receptors on bovine luteal cells.

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Table 1. Effect of biogenic amines on progesterone production (ng/ml culture medium)by dissociated luteal cells in a 2-h incubation.

Treatment	N	0	Treatment concentration 10^{-4}M	10^{-5}M	10^{-6}M	10^{-7}M
Control	14	42.6 ± 3.22				
LH (100 ng/ml)	14	188.3 ± 14.94*				
Acetylcholine	(N)		57.3 ± 6.26(5)	51.5 ± 5.04(8)	59.1 ± 7.28(8)	57.1 ± 4.73(7)
Histamine	4		61.7 ± 13.67	46.9 ± 8.98	44.1 ± 6.80	44.2 ± 5.90
γ-Aminobutyric Acid	4			45.5 ± 4.90	53.0 ± 13.03	47.8 ± 10.86
Dopamine Agonist	4			47.6 ± 3.65	43.4 ± 6.62	44.8 ± 8.07
Melatonin	4			54.9 ± 7.92	54.5 ± 4.76	43.6 ± 4.19
Terbutaline	3		65.3 ± 8.20	58.8 ± 16.49	53.6 ± 7.61	41.5 ± 9.38
Salbutamol	6		69.4 ± 5.66	61.7 ± 5.53	63.8 ± 4.41	49.7 ± 3.28

Values are the mean ± s.e.m.

*P<0.01 compared with control value

Table 2. Effect of biogenic amines on LH-stimulated progesterone production (ng/ml culture medium) by dissociated luteal cells in a 2-h incubation.

Treatment	N	0	Treatment Concentration 10^{-4}M	10^{-5}M	10^{-6}M	10^{-7}M
Control	14	42.6 ± 3.22				
LH (100 ng)	14	188.3 ±14.94*				
Acetylcholine	(5)		191.1 ± 36.96	211.1 ± 27.64	221.1 ± 29.99	197.1 ± 26.08
Histamine	3		156.0 ± 24.29	149.2 ± 21.24	153.4 ± 22.59	149.6 ± 25.19
γ-Aminobutyric Acid	3			168.4 ± 11.85	176.6 ± 13.96	214.8 ± 20.03
Dopamine Agonist	3			198.6 ± 45.88	188.8 ± 46.01	179.9 ± 42.02
Melatonin	3			212.7 ± 10.25	216.1 ± 9.00	212.2 ± 17.16
Terbutaline	3		186.6 ± 18.76	162.8 ± 27.04	148.1 ± 31.05	140.3 ± 24.11
Salbutamol	3		200.6 ± 51.96	203.8 ± 36.28	202.5 ± 48.06	197.7 ± 26.06

Values are the mean ± s.e.m.

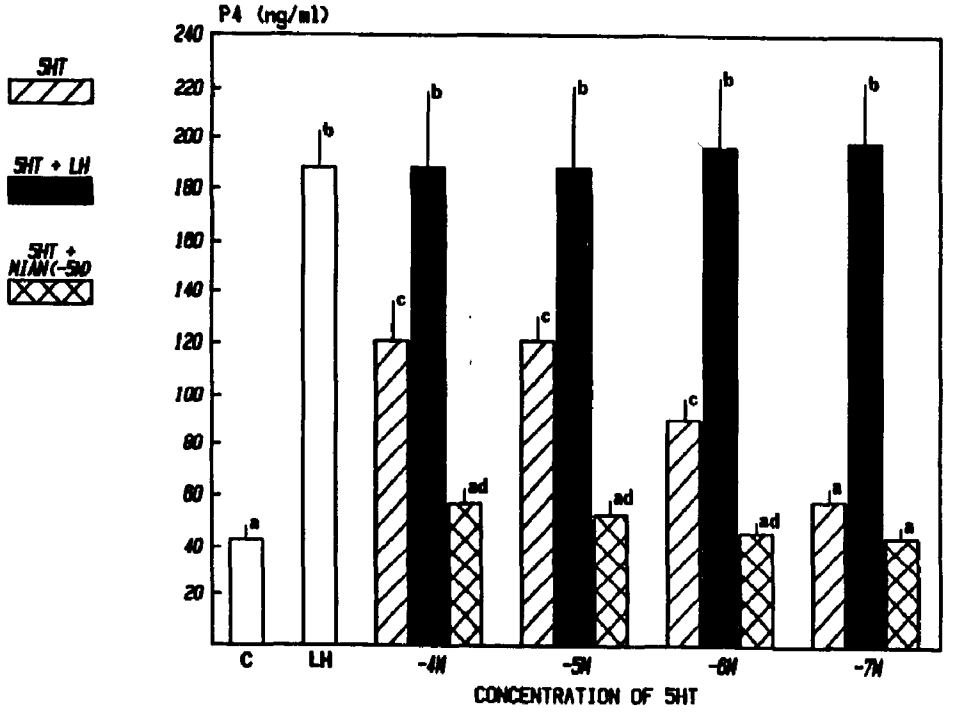
*P<0.01 compared with control value

Figure Legends

Text-fig. 1. Effect of serotonin on progesterone production by dissociated luteal cells. Data are expressed as the mean $\pm$ s.e.m. N = 6(10^{-4}M) and 8(10^{-7}, 10^{-6} and 10^{-5}M) for 5-HT, N = 3 for 5-HT + LH. N = 6 for 5-HT + Mianserin (mian) and N = 14 for control (C) and LH. Bars with different superscript letters are significantly different (P<0.01, LH and 5-HT + LH; P<0.05, 5-HT and 5-HT + mianserin)

Text-fig. 2. Effect of isoproterenol on luteal cell progesterone production. Data are expressed as the mean $\pm$ s.e.m. N=5 (10^{-4}M) and 6 (10^{-7}, 10^{-6} and 10^{-5}M) for isoproterenol (Iso). N = 3 for isoproterenol + LH and isoproterenol + 5-HT, N=14 for control (C) and LH. Bars with different superscript letters are significantly different (P<0.01, LH and ISO + LH; P<0.05 for isoproterenol and isoproterenol + 5-HT).

Text-fig. 3. Effect of β-adrenergic antagonists and mianserin on isoproterenol-stimulated progesterone production. Data are expressed as the mean $\pm$ s.e.m. N = 4 for isoproterenol (Iso) + butoxamine (butox) and isoproterenol + mianserin (mian), N = 3 for isoproterenol + practolol (pract) and isoproterenol + propranolol (propran). Bars with different superscript letters are significantly different from isoproterenol alone (——) (P<0.05, for isoproterenol + propranolol).


5HT
5HT + LH
5HT + NIAN(-5N)
P4 (ng/ml)
240
220
200
180
160
140
120
100
80
60
40
20
a
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c
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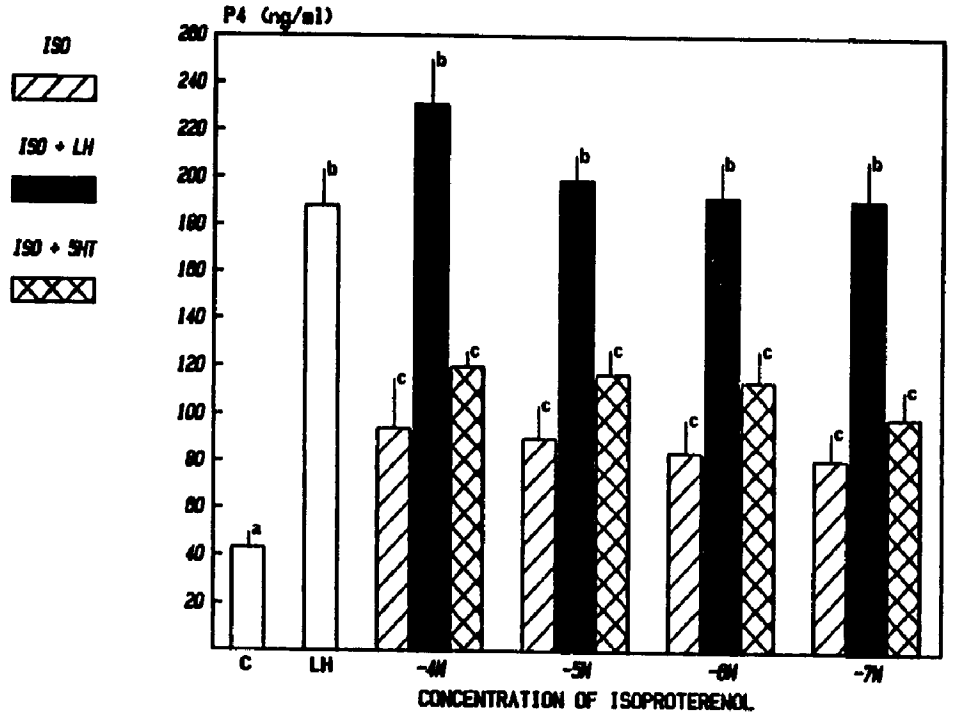
ad
ad
C
LH
-4N
-5N
-6N
-7N
CONCENTRATION OF 5HT


ISO
ISO + LH
ISO + 5HT
P4 (ng/ml)
260
240
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140
120
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a
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LH
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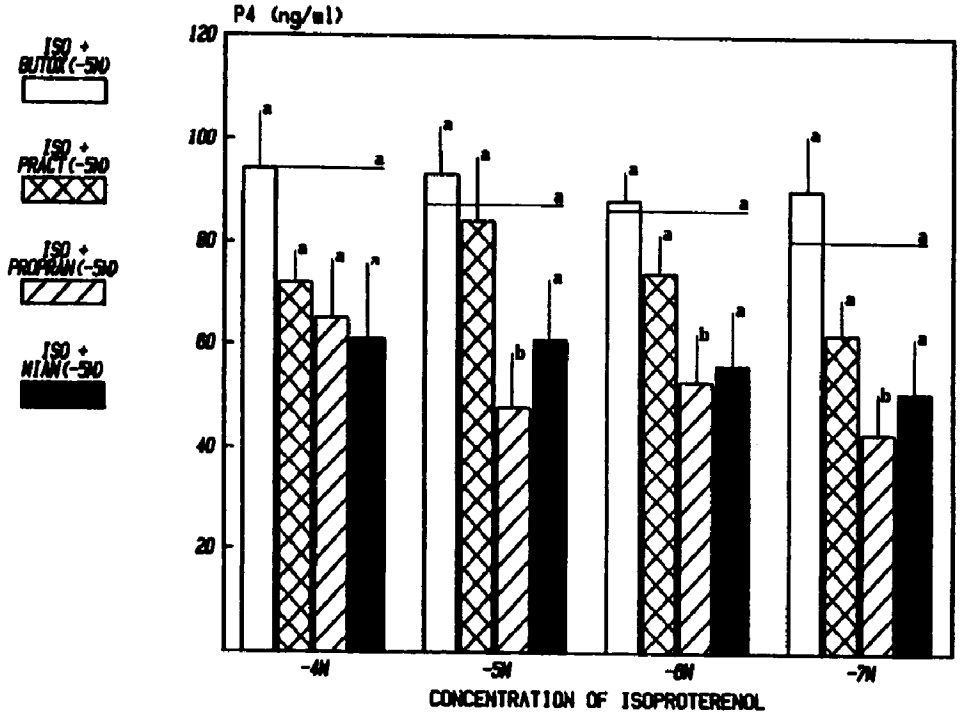
-6W
-7W
CONCENTRATION OF ISOPROTERENOL


ISO + BUTOX(-5N
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P4 (ng/ml)
120
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-4N
-5N
-6N
-7N
CONCENTRATION OF ISOPROTERENOL

EXPERIMENT TWO

Mechanisms involved in the action of serotonin-induced stimulation of progesterone production by bovine luteal cells in vitro

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Summary

Serotonin (5-HT) induced stimulation of progesterone (P_4) production by bovine luteal cells was characterized with respect to a) the receptor subtype mediating this response. b) the steroidogenic response to 5-HT metabolites, c) the role of adenylate cyclase and d) the 5-HT concentration of bovine luteal tissue. Addition of 5-HT (10^{-5}M) stimulated the production of P_4 ($P<0.05$) and this stimulation was inhibited by the 5-HT antagonist mianserin at a concentration of 10^{-5}M ($P<0.05$), but not at a mianserin concentration of 10^{-7}M. Additionally. the response to 5-HT could not be inhibited by ketanserin (10^{-5}M), a 5-HT_2 receptor antagonist. Incubation of luteal cells with a specific 5-HT_1 agonist, DPAT (10^{-4}M), stimulated the production of P_4 ($P<0.05$) and this response could not be blocked by mianserin at 10^{-7}M or by ketanserin. but was inhibited by mianserin at 10^{-5}M ($P<0.05$). The addition of the 5-HT metabolite 5-methoxytryptamine (5-MTA) stimulated P_4 production ($P<0.05$) and this response could be inhibited by mianserin (10^{-5}M, $P<0.05$). Neither N-acetyl-5-HT nor 5-methoxytryptophan significantly affected P_4 production. The addition of the phosphodiesterase inhibitor 3-isobutyl-methylxanthine (IBMX, 0.1mM), potentiated the effects of 5-HT and DPAT ($P<0.05$), but this effect was additive rather than synergistic. In contrast, the addition of luteinizing hormone (10 ng/ml) plus IBMX resulted in a significant synergistic response ($P<0.05$). Analysis of luteal tissue by high performance liquid chromatography showed the concentration of 5-HT to vary with the stage of corpora lutea development, with the highest

concentration being present in early (days 4-6) luteal tissue ($P<0.05$). These results demonstrate the presence of 5-HT within bovine luteal tissue and suggest that the 5-HT and 5-MTA stimulation of P_4 are mediated by a 5-HT_1 receptor which may not be associated with adenylate cyclase.

Introduction

Previous work from this laboratory has shown that serotonin (5-HT) stimulates the production of progesterone (P_4) by dissociated bovine luteal cells in short-term incubation (Battista and Condon. 1986). Additionally. this response could be inhibited by the addition of a 5-HT receptor antagonist, suggesting its action is receptor-mediated. Radioligand binding studies have revealed at least two distinct classes of recognition sites for 5-HT within the central nervous system (Peroutka and Snyder, 1979a). The 5-HT_1 receptor subtype displays a nM affinity for 5-HT and 5-HT agonists and a uM affinity for antagonists. The 5-HT_2 receptor site has the opposite pharmacological profile with a nM affinity for antagonists and a uM affinity for 5-HT agonists (Bennett and Snyder, 1976; Leysen et al.. 1978; Peroutka and Snyder, 1979a). The mechanism of signal transduction for the 5-HT_1 receptor has been suggested to involve the adenylate cyclase system (Peroutka et al.. 1979b, 1981; Barbaccia et al., 1983) while the 5-HT_2 receptor has been found to be associated with phosphatidylinositol hydrolysis and possible protein kinase C activation (Berridge et al.. 1982; Brown et al., 1984; Conn and Sanders-Bush, 1984; Leysen et al. 1984).

The present study further characterized the 5-HT-induced stimulation of P_4 with respect to a) the 5-HT receptor subtype mediating this response, b) the steroidogenic response to 5-HT metabolites and c) the ability of phosphodiesterase inhibition to potentiate the 5-HT response. Additionally, 5-HT concentration in bovine luteal tissue was determined at different stages of luteal development.

Materials and Methods

Materials, drug description and preparation

Serotonin (5-hydroxytryptamine oxalate salt; 5-HT), N-acetyl-5-hydroxytryptamine (N-acetyl-5-HT), 5-methoxy-dl-tryptophan (5-MTP), 5-methoxytryptamine HCl (5-MTA) and 3-isobutyl-methylxanthine (IBMX) were purchased from Sigma Chemical Co. (St. Louis. MO.). Mianserin HCl and (±)-8-hydroxydipropylaminotetralin HBr (DPAT) were from Research Biochemicals, Inc. (Wayland. MA.) and ketanserin was donated by Janssen Pharmaceutica, Inc. (Beerse, Belgium). Luteinizing hormone (LH) was supplied by the National Institutes of Health (NIAMMD-bLH-4). Ham's F12 culture medium was purchased from Grand Island Biological Co. (Grand Island, NY.). The collagenase was Worthington, Type 1, (Freehold, NJ) and N-2-hydroxythylpiperazine-N-2-ethanesulfonic acid (Hepes) was purchased from Calbiochem-Behring (San Diego, CA.).

Mianserin is a nonspecific serotonin antagonist which blocks both 5-HT_1 and 5-HT_2 receptor subtypes depending on concentration (Bennett and Snyder, 1976; Peroutka and Snyder. 1979). Ketanserin is a specific 5-HT_2 receptor antagonist (Leysen et al., 1981, 1982) and DPAT is a specific 5-HT_1 receptor agonist (Hjorth et al.. 1982; Middlemiss and Fozard, 1982). IBMX is a phosphodiesterase inhibitor which acts to elevate intracellular concentrations of cAMP (Butcher and Sutherland, 1962). All hormones and drugs were prepared in cold 0.05M phosphate-buffered saline (pH 7.2) prior to use in each experiment. In all cases n equals the number of animals tested and duplicate determinations of all treatments were performed for each corpus luteum.

Tissue dissociation and incubation

Corpora lutea (CL) were obtained per vaginum from regularly cycling, nonlactating dairy cows on days 10-12 of the estrous cycle (estrus=Day 0). Luteal tissue was placed into Ham's F12 culture medium containing 24mM Hepes (pH 7.4) at 4°C for transport to the laboratory. Luteal tissue was dissociated with collagenase (2000 u/g tissue) and cell number and viability determined as described by Pate and Condon (1982). Incubations were conducted utilizing 2.5×10^5 viable cells in a final volume of 1ml of Ham's F12 culture medium and incubated at 37°C for 2h as previously described (Battista and Condon. 1986).

Progesterone in the incubation medium was quantitated by radioimmunoassy of unextracted samples using antiprogesterone-11-bovine serum albumin (No. 337, Niswender). The progesterone tracer used was [1,2-^{3}H] progesterone (New England Nuclear Boston, MA). The sensitivity of the assay, as determined by the lower 95% confidence limit of the maximum binding in the absence of any unlabeled progesterone, was 0.1ng. The intra-assay and inter-assay coefficients of variability were 6.4% and 11.7%, respectively. All standards were assayed in quadruplicate, and all unknowns were assayed in duplicate.

Experimental procedures

To determine the 5-HT receptor subtype involved in initiating the 5-HT-induced stimulation of P_4, luteal cell suspensions were incubated with 5-HT (10^{-5}M or DPAT (10^{-4}M) either alone or in combination with mianserin (10^{-7}M and 10^{-5}) or ketanserin (10^{-5}M). Control tubes (no additions) were included in each experiment. To evaluate the steroidogenic potential of serotonin metabolites, N-acetyl-5-HT (10^{-5} - 10^{-4}M), 5-MTP (10^{-5} - 10^{-4}M) and 5-MTA (10^{-7} - 10^{-4}M) were added to

incubations and their ability to modulate P_4 production evaluated. To determine if an increase in intracellular cAMP concentration could potentiate the response to 5-HT. luteal cells were incubated with the following treatments in the presence and absence of 0.1mM IBMX: 5-HT (10^{-5}M), DPAT (10^{-4}M), 5-HT + mianserin (10^{-7} and 10^{-5}M), 5-HT + ketanserin (10^{-5}M) and IBMX alone (control).

For the determination of 5-HT concentration. CL were removed per vagium at various stages of the estrous cycle: days 4-6 (early) days 10-12 (mid) and days 15-16 (late). Upon removal, tissue was rinsed with Ham's F12 then cut in half and a cross-sectional slice obtained. The tissue slice was rinsed again and immediately placed on dry ice for transport to the laboratory. Tissue was stored in liquid nitrogen (liquid phase) prior to analysis. Tissue concentrations of 5-HT were determined using high performance liquid chromatography (HPLC) as described by Mayer and Shoup (1983). Briefly, luteal tissue was homogenized in 5% trichloroacetic acid. Dihydroxybenzylamine was added to the tissue prior to homogenization to serve as an internal standard. The homogenate was filtered through a 0.22 micron filter prior to injecting 50 ul aliquots onto an HPLC column. The 5-HT was identified by its retention time compared to an external standard and quantitated against the external standard. The internal standard was used to account for recovery. Recovery from the original homogenate of the internal standard was .90 $\pm$ .02 (mean $\pm$ SEM. n=18).

Statistical analysis

Analysis of differences between treatment means was conducted using one-way analysis of variance followed by the Student-Newman-Keuls mean separation procedure. Two-way analysis of variance was used to test for

a significant interaction in those experiments involving the addition of IBMX.

Results

Incubation of luteal cell suspensions with 5-HT (10^{-5}M) resulted in a significant stimulation of P_4 production ($P<0.05$. Fig. 1). The addition of mianserin at 10^{-5}M but not at 10^{-7}M. inhibited the 5-HT-induced production of P_4 ($P<0.05$). Coincubation of 5-HT with ketanserin (10^{-5}M) did not block the steroidogenic action of 5-HT ($P>0.05$). Addition of DPAT (10^{-4}M) stimulated the production of P_4 ($P<0.05$, Fig.2) and this response could not be inhibited by mianserin (10^{-7}M) or by ketanserin (10^{-5}M) ($P>0.05$) but was blocked by mianserin at 10^{-5}M ($P<0.05$). The combination of 5-HT + DPAT did not result in a further increase in P_4 production above that observed with 5-HT alone (Figs. 1 and 2) but the response to 5-HT + DPAT was greater than DPAT alone ($P<0.05$, Fig. 2).

The effects of 5-HT metabolites on P_4 production are presented in Table 1. Addition of 5-MTA at concentrations from 10^{-6} to 10^{-4}M resulted in a dose-dependent stimulation of P_4 production ($P<0.05$). Mianserin at 10^{-5}M inhibited the 5-MTA response ($P<0.05$). Neither N-acetyl-5-HT nor 5-MTP significantly affected P_4 production at either of the concentrations tested.

Addition of 0.1mM IBMX to the incubation medium increased basal P_4 production (47.4 ± 1.96 vs 103.5 ± 5.42 for control and IBMX, respectively, $P<0.05$, Fig 3). This treatment also significantly potentiated the effects of 5-HT and DPAT, and the potentiation of 5-HT could be blocked by the addition of mianserin at 10^{-5}M ($P<0.05$) but not at a mianserin concentration of 10^{-7}M or by ketanserin ($P>0.05$, Fig. 3). The response to 5-HT was greater than that found for DPAT ($P<0.05$). The

potentiation observed when 5-HT or DPAT was coincubated with IBMX was additive rather than synergistic. In contrast, incubation of luteal cells with LH (10ng/ml) plus IBMX resulted in a significant synergistic response (255.5 + 15.69 vs 358.7 + 18.48 for LH and LH + IBMX, respectively, n=6, $P<0.05$). Neither mianserin nor ketanserin significantly affected basal P_4 production (51.1 ±4.76 and 47.8 ±3.28 for mianserin and ketanserin, respectively, n=6, $P>0.05$).

The results of the HPLC analysis of luteal 5-HT concentration are presented in Fig. 4. Concentration of 5-HT was found to vary significantly with the stage of the estrous cycle ($P<0.05$). 5-HT was in highest concentration in luteal tissue obtained during the early luteal phase and was found to decline as CL development proceeded.

Discussion

The ability of 5-HT to stimulate P_4 production by bovine luteal cells is consistent with our previously reported results (Battista and Condon, 1986). The inability of low molar concentrations of mianserin and the specific 5-HT_2 receptor antagonist ketanserin, to inhibit the action of 5-HT suggests the 5-HT response is mediated by the 5-HT_1 receptor subtype. This conclusion is strengthened by the finding that DPAT, a specific 5-HT_1 agonist, could also significantly stimulate the production of progesterone. Additionally, the action of DPAT could not be inhibited by low molar concentrations of mianserin or by ketanserin but was inhibited by higher concentrations of mianserin.

The addition of the 5-HT metabolite 5-MTA resulted in a dose-dependent stimulation of P_4 similar to that previously reported for 5-HT (Battista and Condon, 1986). The increase in P_4 production in response to 10^{-5}M of 5-HT and 5-MTA was similar. These results suggest 5-MTA is as active as 5-HT in vitro. 5-MTA appears to be acting through the same receptor as 5-HT, since the action of 5-MTA could also be blocked by mianserin. Additional support for this conclusion is drawn from the observations of Bennett and Snyder (1976) who reported 5-MTA was effective in the displacement of [^{3}H] 5-HT binding from cerebral cortex membranes. These authors also reported 5-MTA to be more potent in the displacement of binding from the 5-HT_1 receptor subtype. These results lend further support for a 5-HT_1 receptor site on bovine luteal cell membranes.

In vitro studies utilizing human CL slices showed melatonin stimulated P_4 production, while 5-HT and N-acetyl-5-HT were ineffective

in regulating the production of P_4 (MacPhee et al., 1975). 5-HT has been demonstrated to stimulate hydrocortisone secretion in perfused adrenal gland preparations of the dog (Verdesca et al., 1961). In male rats melatonin and 5-HT inhibit the in vitro biosynthesis of androgens (Peat and Kinson, 1971; Ellis, 1972). We have previously reported that melatonin was unable to alter basal or LH-stimulated P_4 by dissociated bovine luteal cells (Battista and Condon, 1986). In the present study, neither N-acetyl-5-HT nor 5-MTP were effective in regulating steroidogenesis. The inability of N-acetyl-5-HT or melatonin to stimulate P_4 production suggests that the action of 5-HT is not due to its conversions to these metabolites.

The ability of the phosphodiesterase inhibitor IBMX to increase basal and LH-stimulated P_4 production demonstrates our short-term culture system is responsive to increased intracellular concentrations of cAMP. The inability of IBMX to potentiate the response to 5-HT in a synergistic manner may suggest a mechanism of signal transduction not associated with the adenylate cyclase system. These results differ from the 5-HT_1 receptor found within the central nervous system, which has been suggested to involve adenylate cyclase (Peroutka et al., 1979b, 1981; Barbaccia et al., 1983). It is possible that the 5-HT_1 receptor associated with adenylate cyclase suggested by Barbaccia et al. (1983) and the 5-HT_1 receptor found on bovine luteal cell membranes represent different subclasses of the 5-HT_1 receptor which elicit responses through different mechanisms. It is also likely that the 5-HT_1 receptor found on bovine luteal cells is of the 5-HT_{1A} subclass since DPAT has been reported to have a greater affinity for the 5-HT_{1A} receptor site (Middlemiss and Fozard, 1983; Hoyer et al., 1985).

The results of the HPLC analysis demonstrate the presence of 5-HT within bovine luteal tissue and show that the concentration of 5-HT varies with respect to the stage of CL development. In the rat. Clausell and Soliman (1978) have reported 5-HT to be present in whole ovarian homogenates. The concentration of 5-HT was found to vary with the stage of the cycle. with the highest concentrations occurring at estrus. Additionally, 5-HT has been reported in rat testicular tissue (Zieher et al., 1971; Ellis, 1972). Possible sources of luteal 5-HT include mast cells, blood platelets. storage of peripherally circulating 5-HT or de novo synthesis. Histological determination of mast cells using Giemsa, toluidene blue and methylene blue staining techniques has failed to demonstrated the presence of mast cells in either early or mid-cycle luteal tissue (unpublished observations). 5-HT present in luteal tissue may be derived from peripherally circulating 5-HT similar to that reported for rat testicular (Ellis et al., 1972) and adrenal (Verhofstad and Jonsson. 1983) tissues. Although the exact mechanism whereby 5-HT regulates P_4 production is unknown. the ability of 5-HT to stimulate luteal steroidogenesis and the presence of 5-HT within luteal tissue may suggest a physiological role for 5-HT as an intraovarian stimulator of luteal steroidogenesis.

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Table 1. Effect of serotonin metabolites on P_4 production (ng/ml of incubation medium) by dissociated luteal cells in short-term incubation.

		Treatment Concentration				
Treatment	n	0	10^{-4}M	10^{-5}M	10^{-6}M	10^{-7}M
Control	6	43.3 ± 3.14[a]				
5-MTA	6		146.0 ± 7.87[b]	115.5 ± 10.89[c]	93.0 ± 6.98[d]	60.7 ± 2.37[a]
5-MTA + mianserin (10^{-5}M)	4		66.2 ± 2.82[a]	51.0 ± 5.51[a]	50.9 ± 5.83[a]	43.6 ± 4.48[a]
N-Acetyl-5-HT	6		48.6 ± 4.17[a]	52.0 ± 5.57[a]	ND	ND
5-MTP	6		41.8 ± 2.56[a]	44.8 ± 2.71[a]	ND	ND

Values are the mean ± SEM
Numbers with different superscript are significantly different ($P<0.05$).
ND denotes not determined

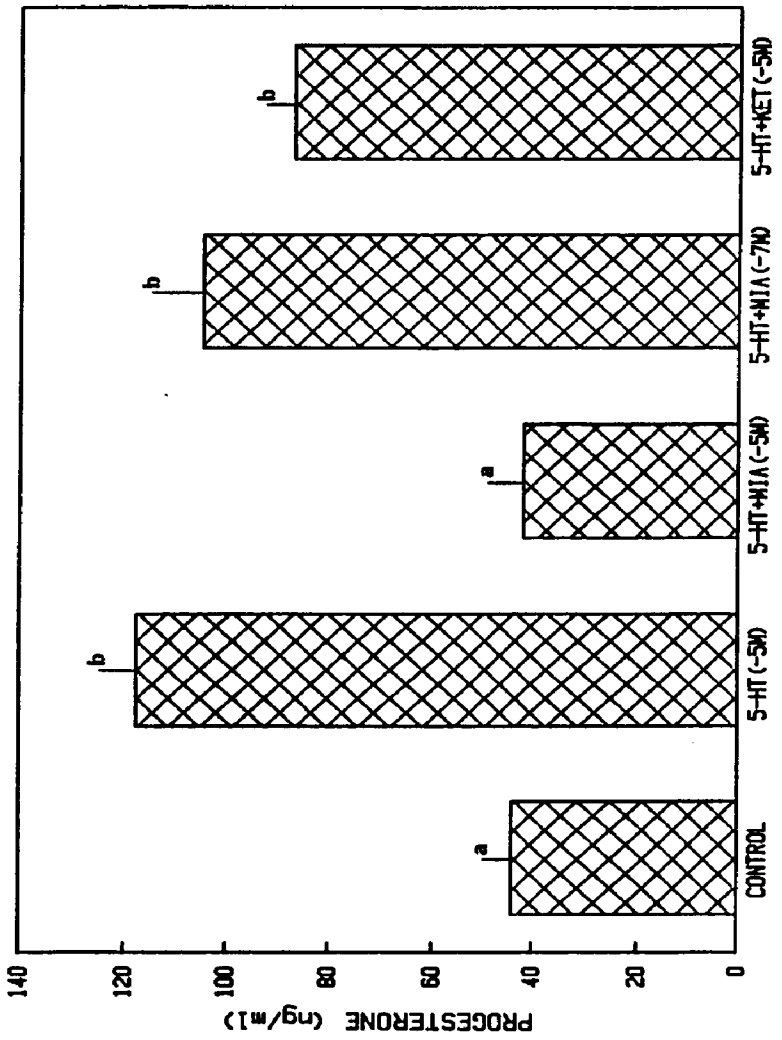
Figure Legends

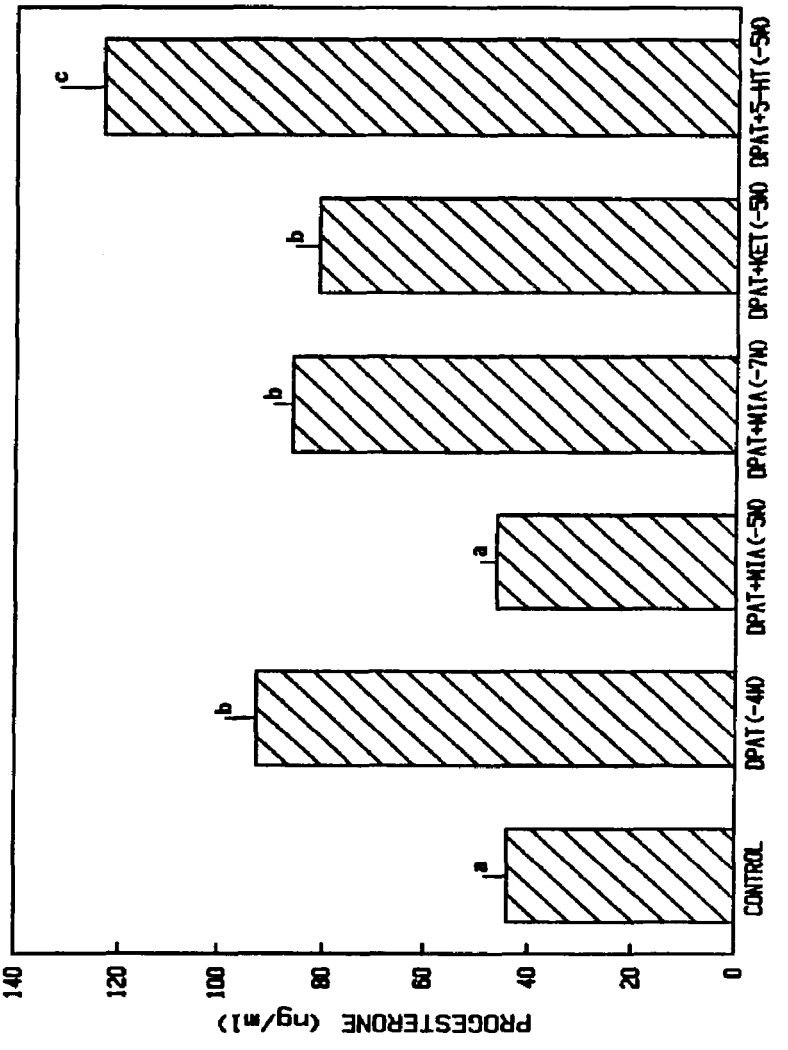
Fig 1. Effect of 5-HT antagonists on 5-HT-induced P_4 production. Data are expressed as the mean ± SEM, n=9 for control and 5-HT, n=6 for all other treatments. MIA=mianserin and KET=Ketanserin. Bars with different superscripts are significantly different (P<0.05).

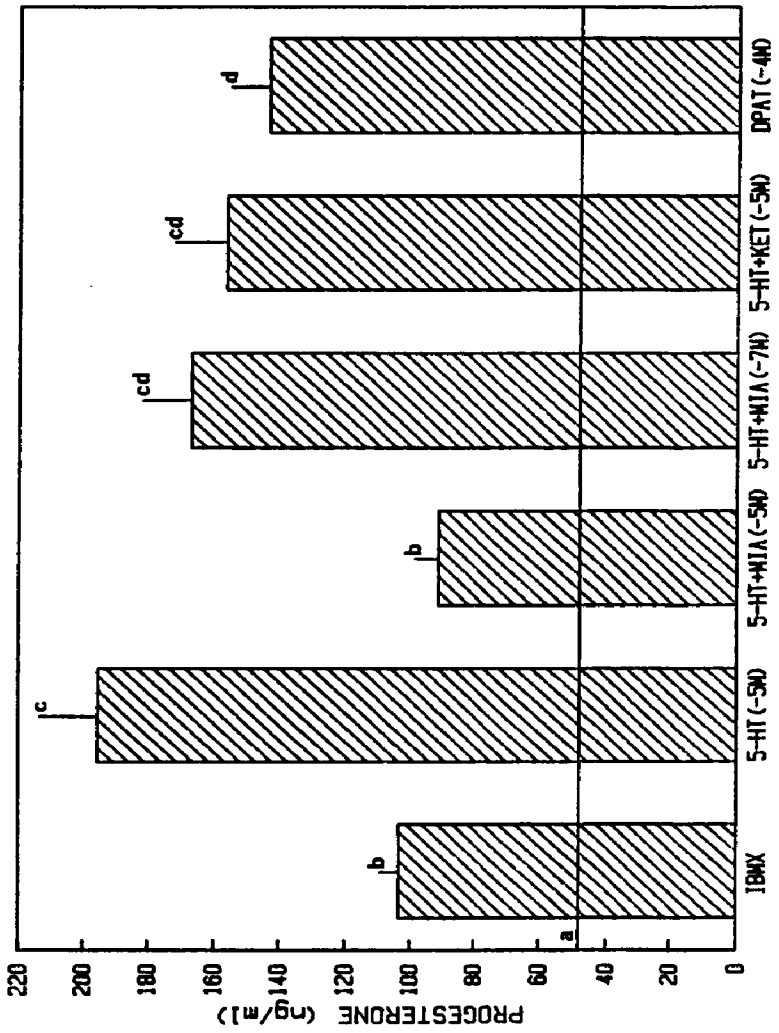
Fig 2. Effect of the 5-HT_1 agonist DPAT on luteal cell P_4 production. Data are expressed as the mean ± SEM, n=9 for control, n=7 for all other treatments. MIA=mianserin and KET=ketanserin. Bars with different superscripts are significantly different (P<0.05).

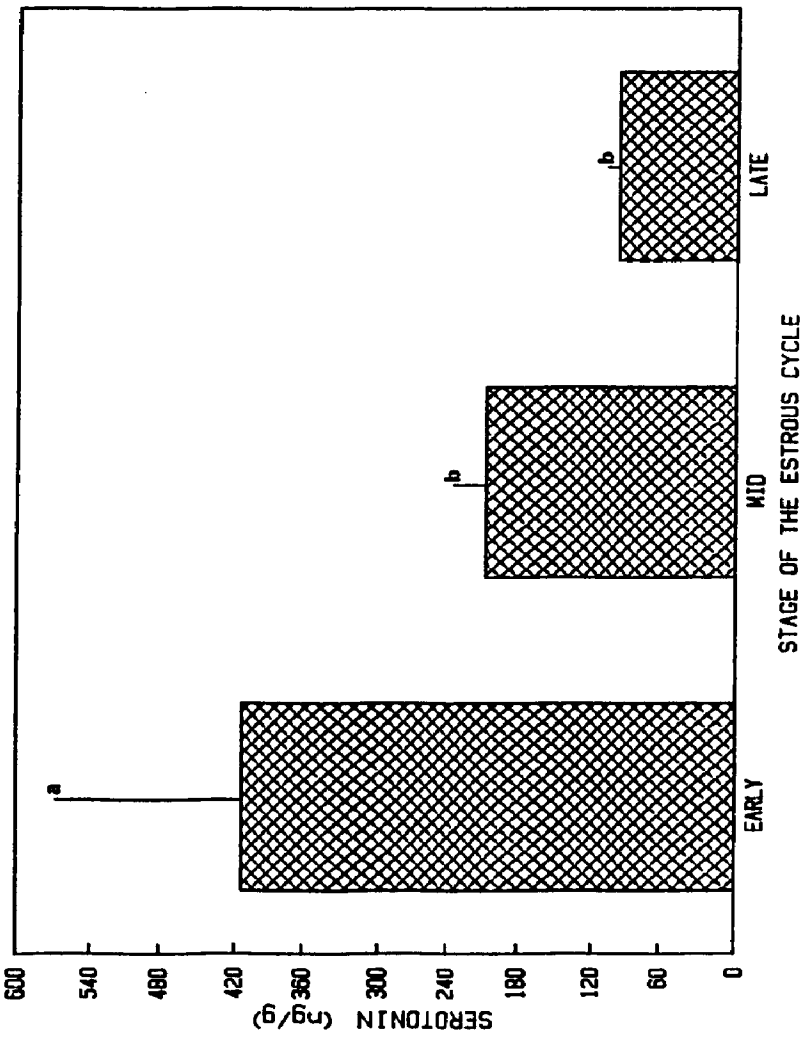
Fig 3. Effect of IBMX on basal P_4 production and P_4 production induced by 5-HT and DPAT either alone or in combination with 5-HT antagonists. Data are expressed as the mean ± SEM, n=6 for all treatments. Solid line denotes control value (no IBMX) of 47.4 ± 1.96 ng/ml. MIA=mianserin and KET=ketanserin. Bars with different superscripts are significantly different (P<0.05).

Fig 4. 5-HT concentration (ng/g of tissue) of bovine luteal tissue as affected by stage of luteotropism. Data are expressed as the mean ± SEM, n=5 for early, n=8 for mid and n=5 for late stages of the cycle. Bars with different superscripts are significantly different (P<0.05).


PROGESTERONE (ng/ml)
140
120
100
80
60
40
20
0
CONTROL
5-HT (-5HT)
5-HT+MIA (-5HT)
5-HT+MIA (-7HT)
5-HT+KET (-5HT)
a
b
b
a
b


PROGESTERONE (ng/ml)
0
20
40
60
80
100
120
140
a
b
a
b
b
c
CONTROL
DPAT(-4N)
DPAT+MIA(-5N)
DPAT+MIA(-7N)
DPAT+KET(-5N)
DPAT+5-HT(-5N)


PROGESTERONE (ng/ml)
220
200
180
160
140
120
100
80
60
40
20
0
a
b
c
b
cd
cd
d
IBMX
5-HT (-5M)
5-HT+MIA(-5M)
5-HT+MIA(-7M)
5-HT+KET(-5M)
DPAT(-4M)


SEROTONIN (ng/g)
600
540
480
420
360
300
240
180
120
60
0
a
b
b
EARLY
MID
LATE
STAGE OF THE ESTROUS CYCLE

EXPERIMENT THREE

A role for alternative pathway catecholamines in the stimulation of steroidogenesis in cow luteal cells

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Summary

Incubation of bovine luteal cells with the alternative pathway catecholamines octopamine, synephrine and deoxyadrenaline at concentrations of 10^{-6} to 10^{-3} M enhanced the production of progesterone ($P<0.05$). Tyramine did not alter basal progesterone production ($P>0.05$). Addition of noradrenaline and adrenaline at concentrations of 10^{-4} to 10^{-7} M significantly elevated the production of progesterone ($P<0.05$). The steroidogenic response to noradrenaline and adrenaline was greater than that for octopamine, synephrine and deoxyadrenaline ($P<0.05$). Response to both primary (10^{-6} M) and alternative (10^{-4} M) pathway catecholamines was inhibited by propranolol (10^{-5} M, $P<0.05$) but not by phentolamine (10^{-5} M, $P>0.05$). These results demonstrate that octopamine, synephrine and deoxyadrenaline can affect steroidogenesis by bovine luteal cells, and their action is mediated by β-adrenergic receptors.

Introduction

The primary catecholamines noradrenaline and adrenaline can stimulate the synthesis *in vitro* of cAMP and progesterone by luteal tissue from a variety of species (cow: Condon & Black, 1976; Godkin, Black & Duby, 1977; Milvae, Alila & Hansel, 1983; rat: Harwood, Dufau & Catt, 1979; Ratner, Sanborn & Weiss, 1980; Norjavaara, Selstam & Ahren, 1982; rabbit: Birnbaumer, Yang, Hunzicker-Dunn, Brockaert & Duran, 1976; ewe: Jordan, Caffrey & Niswender, 1978). Noradrenaline and adrenaline can interact with both α-and β-adrenergic receptors although their stimulatory effects on luteal steroidogenesis are mediated by β-receptors, since their response can be inhibited by β-adrenergic antagonists but not by α-adrenergic antagonists (Condon & Black, 1976; Jordan *et al.*, 1978; Harwood, Richert, Dufau & Catt, 1980; Ratner *et al.*, 1980; Norjavaara *et al.* 1982). In contrast, nothing is known about the catecholamines derived through the alternative catecholamine pathway (Fig. 1) with respect to ovarian steroidogenesis. Octopamine and synephrine have been identified within the central nervous system and peripheral tissues in a number of vertebrate species (Kakimoto & Armstrong 1962; Molinoff & Axelrod, 1969, 1972; Saavedra, 1974; Harmar & Horn, 1976). In peripheral tissues octopamine is associated with sympathetic nerve endings (Molinoff & Axelrod, 1969, 1972) where it can be taken-up, synthesized and stored (Fischer, Musacchio, Kopin & Axelrod, 1964; Kopin, Fischer, Musacchio & Horst, 1964; Kopin, Fischer, Musacchio, Horst & Weise, 1965; Baldessarini & Vogt, 1971). Octopamine accumulates in sympathetic nerves after monoamine oxidase inhibition and is released following neural stimulation (Kopin *et al.*, 1964, 1965;

Fischer, Horst & Kopin, 1965; Baldessarini, 1971; Baldessarini & Vogt, 1972). Additionally, octopamine has a subcellular distribution similar to that of noradrenaline (Musacchio, Kopin & Snyder. 1964; Snyder, Michaelson & Musacchio, 1964; Snyder, Glowinski & Axelrod, 1965; Baldessarini & Vogt 1971; Molinoff & Axelrod, 1972). and a physiological role for octopamine as a cotransmitter with noradrenaline has been proposed (Molinoff & Axelrod 1969, 1972; Axelrod & Saavedra, 1977).

The physiological significance of the alternative pathway cathecholamines with respect to luteal function is unknown. It was the purpose of this study to examine the ability of the alternative pathway catecholamines tyramine, octopamine, synephrine and deoxyadrenaline to stimulate steroidogenesis by bovine luteal cells.

Materials and Methods

Materials, drug description and preparation

Tyramine HCl, DL-octopamine HCl, DL-synephrine. deoxyadrenaline HCl, L-noradrenaline HCl, DL-adrenaline and DL-propranolol HCl were purchased from Sigma Chemical Co., St. Louis, MO. Phentolamine HCl was donated by Ciba-Geigy Corp., Summit, NJ. Ham's F12 culture medium was purchased from Grand Island Biological Co., Grand Island, NY. The collagenase was Worthington, Type 1, Freehold, NJ and N-2-hydroxythylpiperazine-N-2-ethanesulphonic acid (Hepes) was purchased from Calbiochem-Behring, San Diego, CA.

Propranolol is a nonspecific β-adrenergic receptor antagonist and phentolamine is a nonspecific α-adrenergic antagonist. All hormones and drugs were prepared in cold 0.05 M phosphate-buffered saline (pH 7.2) before use in each experiment. In all cases N equals the number of animals tested. Duplicate determination of all treatments was performed for each corpus luteum.

Experimental procedures

Corpora lutea (CL) were obtained per vaginam from regularly cycling, nonlactating dairy cows on Days 10-12 of the oestrous cycle (oestrus=Day 0). Luteal tissue was placed into Ham's F12 culture medium containing 24 mM-Hepes (pH 7.4) at 4^{o}C for transport to the laboratory. Luteal tissue was dissociated with collagenase (2000 u/g tissue) and cell number and viability were determined as described by Pate & Condon (1982).

Incubations were conducted utilizing 2.5 x 10^5 viable cells in a

final volume of 1 ml Ham's F12 culture medium and incubated for 2h at 37°C as previously described (Battista & Condon, 1986). Tyramine, octopamine, synephrine and deoxyadrenaline were added to luteal cell suspensions at a final concentration of 10^{-6} to 10^{-3} M. Noradrenaline and adrenaline were tested at 10^{-7} to 10^{-4} M. For determination of the adrenergic receptor mediating the catecholamine response, noradrenaline and adrenaline (10^{-6} M) or octopamine, synephrine and deoxyadrenaline (10^{-4} M) were added to incubations which had been preincubated for 30 min with propranolol (10^{-5} M) or phentolamine (10^{-5} M).

Progesterone in the incubation medium was quantitated by radioimmunoassy of unextracted samples using antiprogesterone-11-bovine serum albumin (No. 337, Niswender). The progesterone tracer used was [1,2-^{3}H]progesterone (New England Nuclear, Boston, MA). The sensitivity of the assay, as determined by the lower 95% confidence limit of the maximum binding in the absence of any unlabelled progesterone, was 0.1 ng. The intra-assay and interassay coefficients of variability were 7.3% and 12.4% respectively. All standards were assayed in quadruplicate, and all unknowns were assayed in duplicate.

Statistical analysis

Analysis of difference between treatment means was conducted using one-way analysis of variance followed by the Student-Newman-Keuls mean separation procedure.

Results

Luteal steroidogenesis in the presence of alternative pathway catecholamines is shown in Fig. 2. Octopamine, synephrine and deoxyadrenaline significantly enhanced progesterone production at concentrations of 10^{-6} to 10^{-3} M ($P<0.05$). Tyramine was ineffective in influencing the production of progesterone ($P>0.05$). The response to octopamine, synephrine and deoxyadrenaline was similar within each treatment concentration. Addition of noradrenaline and adrenaline at 10^{-7} to 10^{-4} M resulted in a significant elevation in the production of progesterone ($P<0.05$, Fig. 3.). Comparison of the response of luteal cells to primary and alternative pathway catecholamines showed that noradrenaline and adrenaline were more potent agonists than were octopamine, synephrine or deoxyadrenaline ($P<0.05$). The stimulatory effects of noradrenaline and adrenaline (10^{-6} M) and of octopamine, synephrine and deoxyadrenaline (10^{-4} M) were inhibited by propranolol (10^{-5} M, $P<0.05$, Table 1) but not by phentolamine (10^{-5} M, $P>0.05$). Neither propranolol nor phentolamine significantly altered basal progesterone production (data not shown).

Discussion

These results demonstrate a role for the alternative pathway catecholamines octopamine, synephrine and deoxyadrenaline in the stimulation of bovine luteal steroidogenesis. The stimulatory effects of noradrenaline and adrenaline on progesterone production and the ability of the β-adrenergic antagonist propranolol to inhibit this response is consistent with previous results (Condon & Black, 1976; Jordan et al., 1978; Harwood et al., 1980; Ratner et al., 1980; Norjavaara et al., 1982). In other mammalian tissues octopamine has been reported to interact with α and β-adrenergic receptors (Fujiwara Keisuke, Hideo, Takashi & Yoshiko, 1968), exclusively with α-receptors (Kelly & Burks, 1974; Kleinrok, 1979) or exclusively with β-adrenergic receptors (Chiba, 1976). The ability of propranolol but not phentolamine to inhibit the steroidogenic response to octopamine, synephrine and deoxyadrenaline suggests that their action is mediated by β-adrenergic receptors. The lower potency observed for the alternative pathway catecholamines with respect to progesterone production is similar to that reported for their ability to influence other physiological processes (Lands & Grant. 1952; Fujiwara et al., 1968; Nedergaard & Westermann, 1968; Kelly & Burks, 1974).

The mammalian ovary is extensively innervated with adrenergic and cholinergic nerve fibres, although the CL appears to be devoid of any direct neural innervation (Bahr, Kao & Niswender, 1974; Burden, 1978; Stefenson, Owman, Sjoberg. Sporrong & Walles, 1981). No data are presently available to suggest that the bovine CL can synthesize or retain catecholamines. HPLC analysis of bovine luteal tissue did not

reveal noradrenaline or adrenaline to be present in early, mid or late cycle CL (P. Battista, C. Rexroad & W.A. Condon. unpublished observations). The failure to identify these catecholamines in bovine luteal tissue may suggest that the steroidogenic action of octopamine, synephrine and deoxyadrenaline is not due to conversion to primary catecholamines. Additionally, the inability of tyramine to stimulate luteal steroidogenesis may suggest that the enzymes responsible for primary and alternative pathway catecholamine synthesis are not present in bovine luteal tissue.

The mechanism whereby catecholamines stimulate progesterone production in the bovine is unknown. In rat granulosa cells, adrenergic agents increase the enzymic activity of 3β-hydroxysteroid dehydrogenase while decreasing 20α-hydroxysteroid dehydrogenase activity (Hsueh, et al. 1983). Ovarian denervation or chemical sympathectomy decreases 3β-hydroxysteroid dehydrogenase activity in the rat CL, suggesting that cathecholamines may regulate steroidal enzyme activity in vivo (Burden, 1978).

In summary, these results demonstrate that the alternative pathway catecholamines octopamine, synephrine and deoxyadrenaline stimulate in vitro steroid biosynthesis in bovine luteal tissue. The response to these catecholamines is less than that observed for noradrenaline and adrenaline, and the steroidogenic response of octopamine, synephrine and deoxyadrenaline is mediated by β-adrenergic receptors.

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Table 1. Effect of α-and β-adrenergic antagonists on primary and alternative pathway catecholamine-induced progesterone production (ng/ml incubation medium)

	N	Octopamine	Synephrine	Deoxyadrenaline	Noradrenaline	Adrenaline
Agonist alone	6	90.6 ± 2.25[a]	87.4 ± 4.81[a]	96.5 ± 1.92[a]	99.9 ± 6.61[a]	107.2 ± 4.07[a]
Agonist + propranolol	4	57.8 ± 8.54[b]	61.4 ± 5.77[b]	52.3 ± 6.18[b]	55.5 ± 3.30[b]	63.8 ± 6.64[b]
Agonist + phentolamine	4	93.9 ± 4.87[a]	87.9 ± 7.88[a]	89.8 ± 5.42[a]	89.3 ± 6.18[a]	98.7 ± 4.34[a]

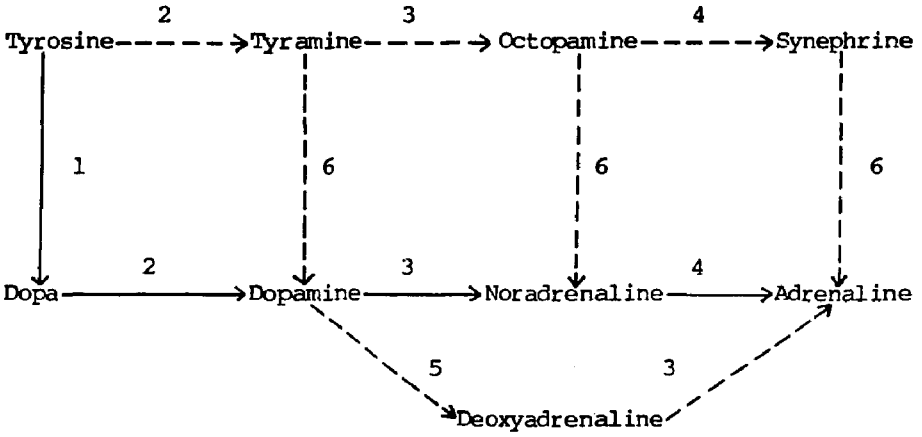
Values are mean ± s.e.m.
Means within columns with different superscript letters are significantly different ($P<0.05$).
Control value equals 46.4 ± 2.13.

Figure Legends

Fig. 1. Primary and alternative pathways for the formation of catecholamines: (1) tyrosine hydroxylase; (2) aromatic aminoacid decarboxylase; (3) dopamine-β -oxidase; (4) phenylathylamine-N-methyl transferase; (5) nonspecific N-methyltransferase; (6) catechol-forming enzyme. Solid lines denote primary pathway. (Taken from Axelrod, 1963; and Cooper, Bloom & Roth, 1982).

Fig. 2. Effect of alternative pathway catecholamines on progesterone production by dissociated bovine luteal cells. Data are expressed as the mean ± s.e.m. (N=6 for all treatments). Solid line denotes control value of 46.4 ± 2.13 ng/ml. Bars within treatments with different superscript letters are significantly different ($P<0.05$).

Fig. 3. Effect of noradrenaline and adrenaline on progesterone production by dissociated luteal cells. Data are expressed as the mean ± s.e.m. (N=6 for all treatments). Solid line denotes control value of 46.4 ± 2.13 ng/ml. Bars within treatments with different superscript letters are significantly different ($P<0.05$).


Tyrosine
Tyramine
Octopamine
Synephrine
Dopa
Dopamine
Noradrenaline
Adrenaline
Deoxyadrenaline
1
2
3
4
5
6
6
6
2
3
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5
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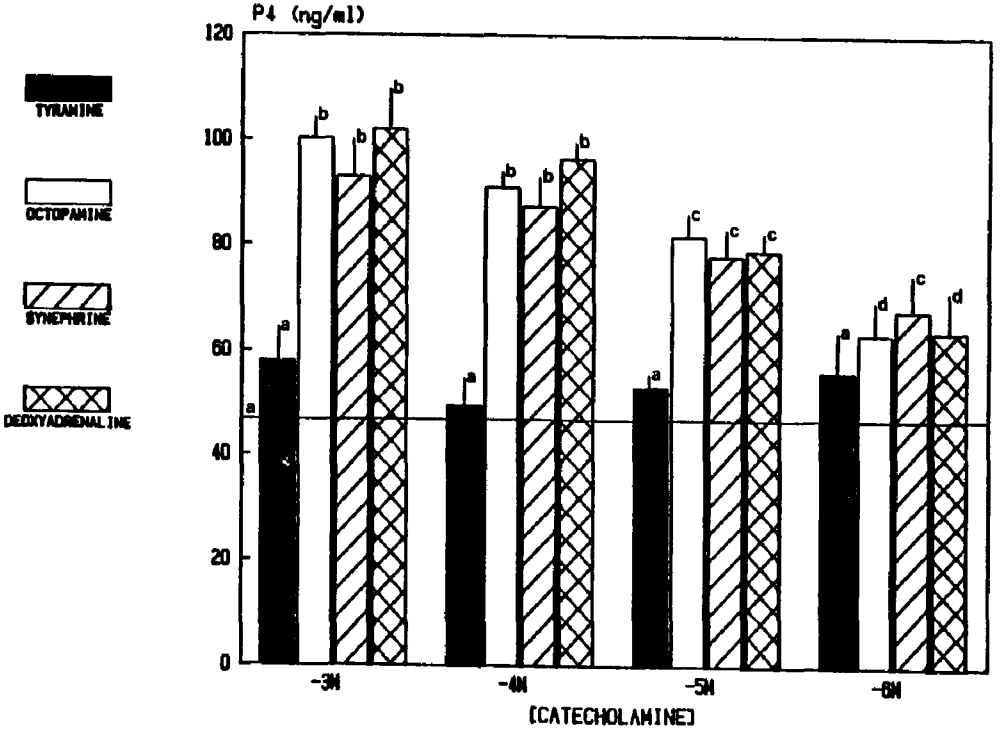
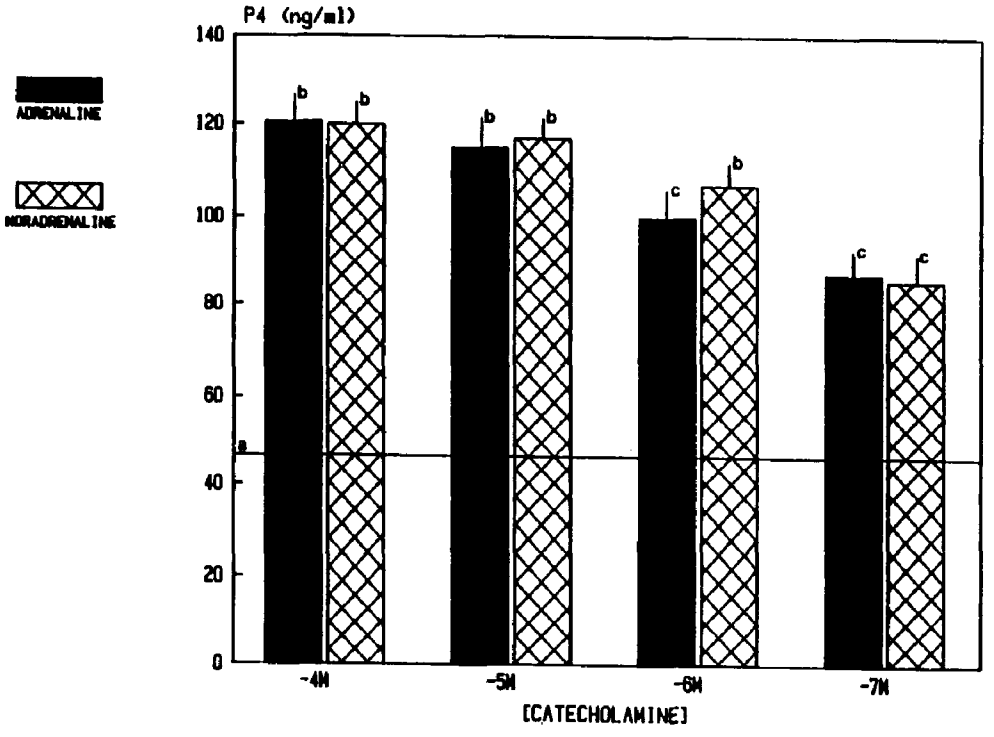

P4 (ng/ml)
120
100
80
60
40
20
0
TYRAMINE
OCTOPAMINE
SYNEPHRINE
DEOXYADRENALINE
-3M
-4M
-5M
-6M
[CATECHOLAMINE]


P4 (ng/ml)
140
120
100
80
60
40
20
0
a
b
b
b
b
c
b
c
c
ADRENALINE
NORADRENALINE
-4M
-5M
-6M
-7M
[CATECHOLAMINE]

EXPERIMENT FOUR

Biogenic amine stimulation of bovine luteal progesterone production *in vivo*

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Summary

The purpose of the present study was to determine if the administration of noradrenaline, adrenaline and serotonin could stimulate luteal progesterone production *in vivo*, as has been observed *in vitro*. Biogenic amines were administered using osmotic pumps placed subcutaneous in the neck region of regularly cycling, nonlactating dairy cows on days 9-11 (oestrus = Day 0) of the oestrous cycle. To monitor the effects of biogenic amines on luteal function, blood samples were collected using indwelling jugular catheters and the plasma fraction analyzed for progesterone. Samples were collected at 4h intervals for the first 12h of treatment and thereafter at 12h intervals for the remainder of the 72h treatment period. Upon administration of varying doses of noradrenaline, adrenaline and serotonin (0.5-2.0ug/kg/h) significant elevation of plasma progesterone concentrations was achieved at a dosage of 2.0 ug/kg/h ($P<0.01$). The response to adrenaline was greater than that observed for noradrenaline and serotonin ($P<0.05$). Within treatment comparison to pretreatment samples showed plasma progesterone concentrations to increase within 4h after the administration of noradrenaline, adrenaline and serotonin ($P<0.05$) and this enhancement was maintained throughout the treatment period ($P<0.05$). The elevation in plasma progesterone concentrations induced by noradrenaline. adrenaline and serotonin was independent of changes in circulating levels of luteinizing hormone. These results support a physiological role for endogenous biogenic amines in the enhancement of bovine luteal progesterone production.

Introduction

The catecholamines, noradrenaline and adrenaline, can stimulate the *in vitro* biosynthesis of cAMP and progesterone by luteal tissue from a variety of species (cow: Condon & Black, 1976; Godkin *et al.*, 1977; Milvae, *et al.* 1983; rat: Harwood *et al.*, 1979; Ratner *et al.*, 1980; Norjavaara *et al.*, 1982; rabbit: Birnbaumer *et al.*, 1976; ewe: Jordan *et al.*, 1978; and sow: Perkins *et al.*, 1986). Recently we reported that the indoleamine serotonin enhances the production of progesterone by bovine luteal cells *in vitro* (Battista & Condon, 1986).

In vivo, the infusion of noradrenaline, adrenaline or the β-adrenergic agonist isoproterenol elevated plasma progesterone concentrations in the ewe (Bolt & Rollins, 1976), and infusion of noradrenaline increased cAMP levels in corpora lutea of the rat (Norjavaara *et al.*, 1983). In contrast, *in vivo* administration of noradrenaline and isoproterenol was ineffective in stimulating progesterone production in oestrous rats, although fenoterol, a β_2-adrenergic agonist, resulted in enhanced progesterone production (Zsolnai *et al.*, 1982). Infusion of adrenaline in early pregnant women increased plasma progesterone (Fylling, 1971a), but these effects may be due to stimulation of placental progesterone production rather than a direct effect on luteal function (Fylling, 1971b; Flint *et al.*, 1974; Csapo & Herczeg, 1977). This is supported by the observation that human luteal tissue is refractory to stimulation by exogenous adrenergic agonists *in vitro* (Richardson & Masson, 1980; Casper & Cotterell, 1984).

The infusion of isoproterenol or the β-adrenergic antagonist propranolol in pseudopregnant rabbits did not alter luteal function

(Gadsby *et al.*, 1985). These authors suggested that endogenous catecholamines are not involved in the *in vivo* stimulation of luteal steroidogenesis. The physiological significance, if any, of biogenic amines in the *in vivo* enhancement of bovine luteal progesterone production is unknown. It was the purpose of this study to determine if the administration of noradrenaline, adrenaline, and serotonin could stimulate the production of progesterone *in vivo*, as has been observed *in vitro*.

Materials and Methods

Experimental Procedures

Serotonin, L-noradrenaline and L-adrenaline (Sigma Chemical Co., St. Louis, MO) were administered using osmotic pumps (Alza Corp., Palo Alto, CA). Pumps were inserted subcutaneously in the neck region of regularly cycling, nonlactating dairy cows on day 9-11 of the oestrous cycle (oestrous = Day 0) under general (Rompun, Miles Laboratories, Inc., Shawnee, KA) and local (Lidocaine HCl, J.A. Webster, Inc., N. Billerica, MA) anaesthesia. Treatments were prepared in a 0.15M NaCl solution (pH 4.0) to deliver controlled release rates of 0.5, 1.0 and 2.0 ug/kg/h. Control animals received osmotic pumps containing NaCl solution only. Blood samples were collected using indwelling jugular catheters (Abbocath- T, 14-gauge x 5 1/2", Abbot Hospitals, Inc., N. Chicago, IL) and samples were collected at 4h intervals for the first 12h of treatment and thereafter at 12h intervals for the remainder of the 72h treatment period. Duplicate pretreatment samples were obtained from each animal by jugular venipuncture prior to experimental manipulation. Blood samples were transferred to heparinized tubes and placed on ice for transport to the laboratory. Samples were centrifuged (4°C) for 20 min at 1.500 x g after which the plasma fraction was removed and stored at -20°C until analyzed for progesterone and luteinizing hormone.

Progesterone present in plasma samples was quantitated after extraction by radioimmunoassay as previously described (Battista *et al.*, 1984). The progesterone antiserum (No. 337, Niswender) was prepared in sheep against 11α-hydroxyprogesterone hemisuccinate

conjugated to bovine serum albumin. The progesterone tracer used was [1,2-^{3}H] progesterone (New England Nuclear, Boston, MA). The sensitivity of the assay as determined by the lower 95% confidence limit of the maximum binding in the absence of any unlabelled progesterone was 0.07ng. The overall recovery of progesterone extracted from plasma was 92 ± 1.5%. The intra- and interassay coefficients of variability were 5.1% and 9.4%, respectively. All samples from an individual animal were analyzed within the same assay and plasma progesterone concentrations are represented as the mean value of duplicate determinations not corrected for recovery. In all cases, n equals the number of individual animals tested.

To assess the effects of chronic administration of biogenic amines on the release of LH, plasma samples were analyzed using a modification of the procedure described by Niswender et al. (1969). Purified ovine LH (LER-1056-C2) was radioiodinated using IODO-GEN (1,3,4,6-tetrachloro-3,6-disphenyl-glycouril- Pierce Chemical Co., Rockville, IL). The primary antibody (antiovine LH, GDN-15) was diluted 1:40,000 in phosphate-buffered saline (0.14M NaCl, 0.01M $NaPO_4$, pH 7.4) containing 2% normal rabbit serum. Second antibody precipitation was achieved using sheep-anti-rabbit sera (DBRC-1) diluted 1:8 in phosphate-buffered saline. Precipitation of the second antibody complex was facilitated using 10% polyethylene glycol. Plasma samples were assayed in duplicate using 100ul of plasma. The mean of duplicate values was used for each unknown and all samples were analyzed within one assay. The sensitivity of the assay averages 0.2 ng. The minimal detectable value was used for samples with nondetectable LH concentration. The intra-assay coefficient of variation was 9.5%.

Statistical Analysis

Differences between treatment means were evaluated using one-way analysis of variance and Student-Newman-Keuls mean separation procedure. Within treatments, differences were determined using paired t-test.

Results

The effect of biogenic amines on mean plasma progesterone concentration over the 72h treatment period are shown in Table 1. The administration of noradrenaline, adrenaline and serotonin at 0.5 and 1.0 ug/kg/h did not significantly alter plasma progesterone concentrations from those observed in animals receiving no treatment ($P>0.05$). Administration of biogenic amines at 2.0 ug/kg/h significantly elevated circulating levels of progesterone above those in nontreated animals ($P<0.01$). The response to adrenaline was greater than that found for noradrenaline and serotonin ($P<0.05$). The time-course response to biogenic amine stimulation is shown in Fig. 1. Administration of noradrenaline and adrenaline elevated plasma progesterone within 4h ($P<0.05$, Fig. 1a), and this stimulation was maintained throughout the treatment period ($P<0.05$). Similarly treatment with serotonin significantly elevated progesterone within 4h ($P<0.05$. Fig. 1b), and this elevation was likewise maintained throughout the 72h treatment period ($P<0.05$).

None of the biogenic amines tested significantly altered circulating levels of LH. Mean plasma LH concentrations over the 72h treatment period for noradrenaline, adrenaline, serotonin and control animals were 0.22 ± 0.11, 0.22 ± 0.12, 0.22 ± 0.11 and 0.26 ± 0.13 ng/ml, respectively ($P>0.05$).

Discussion

These results demonstrate a role for noradrenaline, adrenaline and serotonin in the *in vivo* stimulation of bovine luteal progesterone production. None of the biogenic amines tested significantly altered circulating levels of LH, suggesting a direct effect of these amines on the corpus luteum. These results are consistent with the finding that none of the biogenic amines used cross the blood-brain barrier (Axelrod *et al.*, 1959; Douglas. 1970) or significantly alter pituitary LH release under acute conditions (Kamberi & McCann, 1969; Schneider & McCann. 1969; Kamberi *et al.*. 1970; Blake, 1976). These results support the *in vitro* stimulatory effects of noradrenaline, adrenaline (Condon & Black, 1976; Godkin *et al.*. 1977; Milvae *et al.*. 1983) and serotonin (Battista & Condon. 1986a) on bovine luteal progesterone production. The *in vivo* enhancement of luteal function by noradrenaline and adrenaline reported in the present study supports previous results obtained in the ewe (Bolt & Rollins, 1976) and rat (Norjavaara *et al.*, 1983).

Although the mammalian ovary has both adrenergic and cholinergic nerve fibres, the corpus luteum does not appear to be directly innervated by either neural system (Bahr *et al.*, 1974; Burden. 1978; Stefenson *et al.*, 1981). Support for a physiological role of the nervous system in the control of ovarian steroidogenesis is suggested by the finding that electrical stimulation of specific brain regions increased the concentration of estradiol and progesterone in ovarian venous blood of hypophysectomized-adrenalectomized. pro-oestrous rats (Kawakami *et al.*, 1981). Sectioning of the superior ovarian nerve decreased estradiol and progesterone concentrations in ovarian venous

blood of pro-oestrous rats (Aguado & Ojeda. 1984) while electrical stimulation of the superior ovarian nerve enhanced luteal progesterone production in dioestrous rats through stimulation of β -adrenergic receptors (Weiss *et al.*,1982).

In addition to the absence of direct adrenergic innervation, we have been unable to detect noradrenaline or adrenaline in bovine luteal tissue using HPLC analysis (Battista & Condon, 1986b). In contrast, we have found serotonin in bovine luteal tissue (Battista & Condon, 1986a). Possible sources of luteal serotonin include mast cells, blood platelets, storage of peripherally circulating serotonin or de novo synthesis. Histological determination of mast cells using Giemsa, toluidene blue and methylene blue staining techniques has failed to demonstrate the presence of mast cells in either early or mid-cycle luteal tissue (unpublished observations). Serotonin present in luteal tissue may be derived from peripherally circulating serotonin similar to that reported for rat testicular (Ellis *et al.*, 1972) and adrenal (Verhofstad & Jonsson, 1983) tissues. We are presently conducting immunohistochemical experiments to examine these possibilities and to determine the cellular location of serotonin. The mechanism whereby biogenic amines enhance bovine luteal progesterone production is unknown. *In vitro* studies using rat granulosa cells showed primary catecholamines increased the enzymatic activity of 3β -hydroxysteroid dehydrogenase while decreasing 20α -hydroxysteroid dehydrogenase activity (Hsueh *et al.* 1983). Ovarian denervation or chemical sympathectomy decreases 3β-hydroxysteroid dehydrogenase activity in both the interstitial gland and corpus luteum of pregnant rats (Burden & Lawrence, 1977).

The ability of the primary pathway catecholamines noradrenaline and adrenalineto stimulate luteal progesterone production both *in vivo* and *in vitro* suggests a physiological role for endogenous adrenergic control of bovine luteal function. Additionally, the ability of serotonin to enhance the *in vivo* and *in vitro* production of progesterone and the presence of serotonin within bovine luteal tissue suggests a physiological role for serotonin as an intraovarian stimulator of luteal function.

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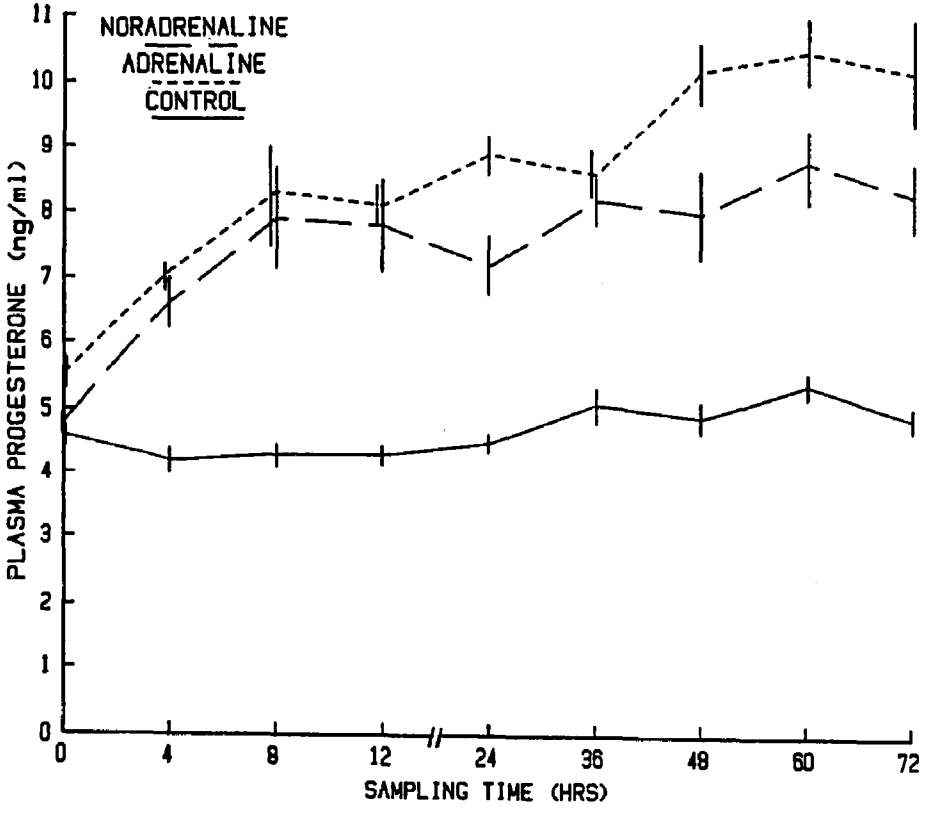
TABLE 1: Effect of biogenic amines on mean plasma progesterone concentrations (ng/ml) over a 72h treatment period.

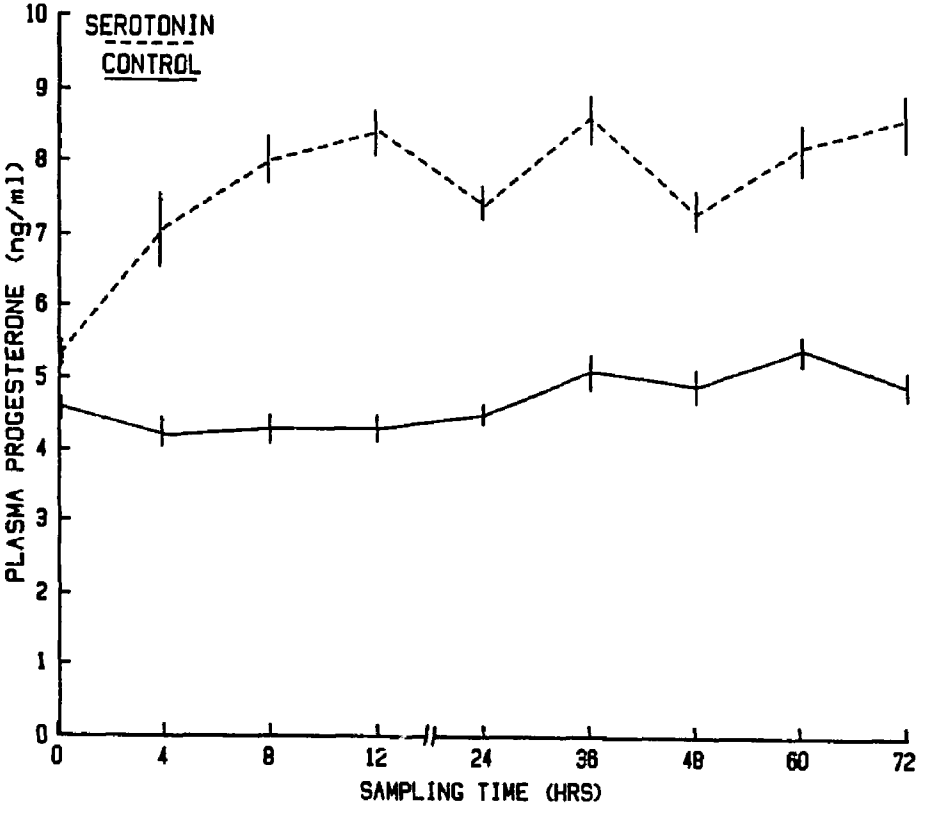
Biogenic Amine	N	Concentration (ug/kg/h)	Plasma Progesterone (mean ± s.e.m.)
Noradrenaline	2	0.5	4.0 ± .37[a]
	2	1.0	4.8 ± .40[a]
	4	2.0	7.9 ± .26[b]
Adrenaline	2	0.5	4.7 ± .60[a]
	2	1.0	5.1 ± .62[a]
	4	2.0	9.0 ± .46[c]
Serotonin	2	0.5	4.5 ± .17[a]
	2	1.0	4.4 ± .29[a]
	5	2.0	7.9 ± .23[b]
None	4	0	4.2 ± .17[a]

N equals the number of individual animals tested. Means with different superscript letters are significantly different ($P<0.01$ for noradrenaline, adrenaline and serotonin vs. control, $P<0.05$ for adrenaline vs. noradrenaline and serotonin).

FIGURE LEGEND

FIGURE 1. Time-course effect of (a) noradrenaline, adrenaline and (b) serotonin on the *in vivo* stimulation of bovine luteal progesterone production. Values are the mean + s.e.m., N = 4 for noradrenaline, adrenaline and control, N = 5 for serotonin. All treatment values are significantly different from corresponding time 0 values ($P<0.05$).


NORADRENALINE
ADRENALINE
CONTROL
PLASMA PROGESTERONE (ng/ml)
0
1
2
3
4
5
6
7
8
9
10
11
0
4
8
12
24
36
48
60
72
SAMPLING TIME (HRS)


SEROTONIN
CONTROL
PLASMA PROGESTERONE (ng/ml)
SAMPLING TIME (HRS)
0
1
2
3
4
5
6
7
8
9
10
0
4
8
12
24
36
48
60
72

SUMMARY AND SIGNIFICANCE OF RESULTS

Catecholamine Studies

1) The enhancement of bovine luteal P_4 production in response to catecholamines is mediated by a mixed population of β-adrenergic receptors. Both β_1- and β_2-adrenergic receptors are present on bovine luteal cell membranes.

2) The catecholamines: octopamine, synephrine and dexoyepinephrine. which are dervived through an alternative or secondary pathway. stimulated basal P_4 production. The stimulatory effect of these amines was mediated by β-adrenergic receptors.

3) HPLC analysis of bovine luteal tissue did not demonstrate detectable levels of either NE or E in luteal tissue obtained at various stages of the estrous cycle.

4) The *in vivo* administration of NE and E stimualted bovine luteal function, as assessed by elevated levels of plasma P_4. The increase in circulating levels of P_4 in response to NE and E treatment was independent of changes in circulating levels of LH.

Biogenic Amine Studies

1) The addition of 5-HT resulted in a dose-dependent enhancement of P_4

production by dissociated bovine luteal cells in short-term incubation.

2) Incubation of luteal cells with the 5-HT metabolite 5-MTA, also stimulated the production of P_4.

3) The response to 5-HT was mediated by a 5-HT_1 receptor which may not be associated with adenylate cyclase.

4) Analysis of bovine luteal tissue by HPLC demonstrated the presence of 5-HT and showed that the concentration of 5-HT varied with the stage of luteal development.

5) Administration of 5-HT in vivo resulted in elevated levels of plasma P_4. The change in plasma P_4 was independent of alterations in circulating levels of LH.

The ability of catecholamines to stimualte luteal steroidogenesis both in vitro and in vivo supports a physiological role for endogenous catecholamines in the stimulation of bovine luteal function. The inability to detect NE and E with bovine luteal tissue suggests that the bovine CL is unable to synthesize or store catecholamines. Thus, neurotransmitter input to the CL may arise from the sympathetic neural network present within the ovary or from circulating catecholamines. Direct experimentation of catecholamine synthesis by luteal tissue has not been conducted. Further research is required to address this question. and to determine the significance of innervation to the normal functioning of the bovine CL.

However, based on the results of the present studies it would be anticipated that inhibition of neural input to the CL would result in luteal insufficiency. Thus intraovarian infusion of a β-adrenergic antagonist such as propranolol, should result in reduced circulating levels of P_4, thereby attributing physiological significance to neural stimulation in the normal functioning of the bovine CL. It could be speculated that a deficiency in neural input to the CL may in part account for pregnancy failure. Since successful conception is dependent upon adequate levels of P_4, insufficient neural input might explain the lower levels of P_4 associated with some repeat-breeder dairy cattle.

Additionally, neural enhancement of bovine luteal function may be involved in explaining reproductive insufficiency resulting from stress. Stress factors such as extremes in temperature, milk production, nutritional deprivation, or excessive physical exercise may result in enhanced neural stimulation of luteal steroidogenesis. As a result, circulating levels of P_4 would be elevated above and beyond normal limits. Prolonged exposure to P_4 would then alter steroid feedback mechanisms at the hypothalamus and pituitary, thereby inhibiting reproductive cyclicity.

The ability of 5-HT to stimulate luteal P_4 production both *in vitro* and *in vivo*, coupled with the presence of 5-HT within bovine luteal tissue, is supportive of a physiological role for endogenous 5-HT as an intraovarian stimulator of luteal function. Further studies are required to directly access the serotonergic contribution to normal luteal function, to determine the cellular location of 5-HT within the CL, and to assess the capabilities for *de novo* synthesis.

With respect to physiological significance, the higher concentration

of 5-HT present in newly formed luteal tissue may suggest a role for 5-HT in the process of luteinization. 5-HT may also play a luteotropic role during early CL formation, a time at which LH is ineffective in stimulating P_4 production.

It would be anticipated based on the results of the present studies that the administration of a 5-HT antagonist such as mianserin, would reduce luteal P_4 secretion. Such an experiment would demonstrate more directly the serotonergic contribution to normal luteal function. If 5-HT antagonists exhibit luteolytic properties, such a pharmacological tool may lead to better methods for the induction of luteolysis, and thus better strategies for estrous cycle control.

Lastly, 5-HT may be involved in early pregnancy recognition. Based on the finding that embryos can secrete platelet activating factor and that platelets contain 5-HT. It is possible that embryo-derived platelet activating factor may act on platelets within the CL to stimulate the release of 5-HT, which would then stimulate P_4 secretion by the maternal CL. Thus, identification of the mechanisms controlling early pregnancy recognition may prove helpful in preventing early embryonic death.

In summary, the results of these studies support the concept that biogenic amines contribute to the physiological control of bovine luteal function. In the future these results may lead to new areas of chemical control of luteal function, which may result in better synchronization strategies for breeding and embryo transfer.